



# **The Role of Heparan Sulfates in Fibroblast Growth Factor Action**

A thesis submitted for the degree of Doctor of Philosophy  
at the Australian National University

by

**Kathryn Brown**

Cell-Cell Interaction Group  
Division of Cell Biology  
John Curtin School of Medical Research  
Australian National University

September 1993

## Acknowledgements

I would like to thank my supervisor, Dr. David Ornitz, for his guidance, support, and encouragement throughout this project. I would also like to thank my friends and family for their support and encouragement.

My sincere thanks to Dr. David Ornitz, for his guidance, support, and encouragement throughout this project.

## Statement

I certify that, except for the experiments presented in section 4.2.7 and Figures 4.8 and 4.9 which were performed by Dr David Ornitz, all experiments described in this thesis represent my own work and have not previously been submitted for a degree at this, or any other university.

*Kathryn J. Brown*

Kathryn J.I. Brown  
September 1993

Many thanks to my supervisor, Dr. David Ornitz, for his guidance, support, and encouragement throughout this project.

I am most grateful to my mother and father for their guidance, support, and encouragement throughout this project.

A very special thanks to David for his guidance, support, and encouragement throughout this project.



## Acknowledgements

I would like to thank my supervisor, Dr Chris Parish, for his enthusiastic supervision of my experimental and written work and for his willingness to discuss it at any time.

My warmest thanks to Dr Ian Hendry for being an invaluable friend and advisor.

I would also like to thank Dr Craig Freeman for helpful advice during the isolation of HSPGs, Dr John Morisson for doing the scatchard analyses of the data, Mr Colin McLachlan for his generous help with computing, Dr Michael Crouch for supplying me with BALB/c 3T3 cells, and Dr Arnie Leon for help with the curve fitting program.

I must also thank the photography unit at JCSMR, particularly Mr Marc Fenning for his excellent help with the Macintosh computer, and Mr Geoff Osborne and Ms Sabine Gueninger for their help with the FACScan.

My thanks must also go to Ms Karen Jakobsen for technical help and to Ms Susan Maynes for providing me with HUVE cells and extracellular matrices.

Many thanks to my fellow students, Mark Bartlett and Lyz Hindmarsh for being such good company and fun.

I am most grateful to my Mother and Father for their continuous love and support.

A very special thanks to Alistair for always being my loving friend.

## Abstract

The central event in growth factor action is the binding of the growth factor to the signal transducing receptor on the plasma membrane of target cells. Fibroblast Growth Factors (FGF) bind to specific (high affinity) receptors that possess protein tyrosine kinase activity in their cytoplasmic domain and are directly involved in FGF signal transduction. Furthermore, as is being discovered for an increasing number of growth factors and cytokines, they also bind to cell surface heparan sulfate proteoglycans (HSPGs) which are indispensable accessories for the binding of the growth factor to the signal transducing receptor.

In this thesis, I have attempted to study the role of HSPGs in the interaction of FGFs with their signaling receptor, FGFR. In particular, I have examined why bFGF is more potent than aFGF *in vitro*, why the mitogenic activity of aFGF in cell culture is potentiated by heparin while that of bFGF is generally not, and the mechanism by which this potentiation occurs. Comparative studies were performed with both acidic and basic FGF in an attempt to further understand these phenomena and the possible involvement of heparan sulfates. An understanding of the requirements for the interaction between FGFs and heparan sulfate (HS) chains, and of how acidic and basic FGF activity is modulated by HSPGs, may facilitate the development of compounds which disrupt this interaction, thereby inhibiting the interaction of FGF with its signaling receptors. Such inhibitors of FGF action could be important in the inhibition of angiogenesis which becomes a pathological process during tumour formation and many other diseases.

In chapter 3, several aspects of the interaction of FGFs with HSPGs were examined. First, reciprocal cross blocking studies demonstrated that aFGF and bFGF bind to identical or closely associated heparan sulfate motifs on



BALB/c 3T3 cell surface HSPGs. However, the binding affinity of the two growth factors for these HSPGs differs considerably, binding-inhibition data indicating that aFGF has a 4.7 fold lower affinity than bFGF for 3T3 HSPGs. Studies of the dissociation kinetics of aFGF and bFGF demonstrated that bFGF dissociates from the FGFR at least 10-fold slower than aFGF ( $t_{1/2}$  >250min versus 25 min) whereas, following removal of cell surface HSPGs by heparinase treatment, the dissociation rate of both FGFs is similar and rapid ( $t_{1/2}$  12min versus 7min). These results support the concept that cell surface HSPGs stabilize the interaction of FGFs with FGFR, possibly by the formation of a ternary complex. The lower binding affinity of aFGF for HSPGs, compared with bFGF for HSPGs, appears to result in aFGF binding to the FGFR being less effectively stabilized by HSPGs. Such a model may explain the lower mitogenic activity of aFGF compared with bFGF for 3T3 cells and suggests that heparin usually potentiates aFGF-induced, but not bFGF-induced, mitogenesis because of aFGF's relatively low affinity for cell surface HSPGs. In this context, heparin had no effect on the rate at which acidic and basic FGF dissociated from FGFR but it did enhance the net binding of aFGF and bFGF to FGFR on cells from which HSPGs had been removed by treatment with heparinase. These data are discussed in the context of the ability of heparin/HSPGs to promote FGF binding to FGFR by inducing a conformational change in the growth factor and/or by enabling the formation of a ternary complex.

The studies described in chapter 4 are primarily based upon the finding by Belford et al.,(1992) that carboxyl-reduced heparin (CR-heparin), despite binding aFGF as effectively as native heparin, is much less potent at augmenting aFGF-induced mitogenesis. The chapter describes experiments which examined this phenomenon in more detail in the hope that it would shed light on the mechanism by which heparin potentiates aFGF activity.



Initial studies confirmed the finding of Belford et al.,(1992) and proteolysis protection experiments revealed that CR-heparin was as effective as native heparin at protecting aFGF from proteolytic degradation. In contrast, CR-heparin was shown to be considerably less effective than native heparin at enhancing the binding of aFGF to either the FGFR on 3T3 cells or to a soluble form of recombinant murine FGFR1. Furthermore, CR-heparin only bound to a subset (approx. 1/3) of heparin receptors on 3T3 cells and bound weakly to a heparin binding site on FGFR1. Since CR-heparin protected aFGF from proteolysis and bound aFGF with high affinity it appeared unlikely that the inability of CR-heparin to enhance aFGF binding to the FGFR was due to its failure to induce a conformational change in aFGF essential for FGFR binding. In contrast, the reduced ability of CR-heparin to interact with heparin binding sites suggests that it is much less efficient than heparin at facilitating the formation of a ternary complex between aFGF and the FGFR. Such a ternary complex would require a heparin-FGF complex to crosslink the FGFR with a heparin binding site either on the FGFR itself or on adjacent molecules on the cell surface.

Additional studies demonstrated that CR-heparin interacted with bFGF with a binding affinity comparable to native heparin. Nevertheless, unlike native heparin, CR-heparin was completely unable to restore binding of bFGF to soluble recombinant FGFR1. However, CR-heparin was quite an effective inhibitor of native heparin-induced binding of bFGF to FGFR1. Such results were not obtained with aFGF. These observations suggest that acidic and basic FGF have different requirements for binding to FGFR1 and a model has been proposed to explain these differences.

In chapter 5, three major heparin-binding proteins, histidine-rich glycoprotein (HRG), antithrombin III (ATIII) and platelet factor 4 (PF4), were tested for their ability to act as modulators of FGF activity by competing with the FGFs for



binding to cell surface HSPGs. HRG from both chicken and human, and human PF4 were demonstrated to compete with each other and with acidic and basic FGF for binding to BALB/c 3T3 cell surface HSPGs, whereas ATIII did not compete. Thus HRG, PF4, aFGF and bFGF all interact with the same HS chains on the 3T3 cell surface, either binding to the same or to adjacent saccharide sequences on the chains. In terms of their relative binding affinity for cell surface HSPGs, the hierarchy was shown to be  $PF4 \geq bFGF > aFGF = cHRG > hHRG$ . HRG was also shown to bind to extracellular matrices (ECMs), originating from bovine corneal endothelial cells, in a heparin-inhibitable manner. Indeed both HRG and PF4 were shown to effectively inhibit the binding of  $^{125}I$ -aFGF and  $^{125}I$ -bFGF to ECMs at concentrations thought to occur *in vivo*. Based on these findings, it is proposed that HRG and PF4 may act as positive regulators of FGF activity by displacing FGF from the ECM or basement membrane and making FGF available to responsive cells. Alternatively, they could act as negative regulators by masking HSPGs on responsive cells and preventing FGFR activation.

In conclusion, the results presented in this thesis confirm the importance of HSPGs in the interaction of FGFs with the FGFR. It appears that the reason for the difference in potency of aFGF and bFGF is due to the lower binding affinity of aFGF for the 3T3 cell surface HSPGs which results in the formation of fewer ternary complexes between aFGF, HSPGs and FGFR. Basic FGF, by having a higher affinity for cell surface HSPGs is able to activate FGFRs at much lower concentrations than aFGF. Similarly, the reason why heparin potentiates aFGF-induced activity but generally not bFGF-induced activity, appears to be due to aFGF's ability to bind heparin better than HSPGs and to utilize heparin more effectively in the formation of productive signaling complexes with the FGFR. Thus it appears, given the correct FGFR is expressed on the cell surface, FGF mitogenic activity is directly related to the affinity of the growth factor for cell surface HSPGs. The ability of



physiologically relevant concentrations of HRG and PF4 to inhibit the binding of acidic and basic FGF to HSPGs present both on the cell surface and in the ECM suggests that they may act as regulators (particularly HRG) of FGF activity *in vivo*.

ATM

ATP

BFGF

BPI

BSA

BSB

cAMP

CHAPS

CHRU

CPM

CR-hyaluron

CPL

DMSO

EG

E11

ECGF

ECM

EDTA

FACS

FCS

FGF

FGFR

FITC

FU

FM

FM1A2

GAG

GdMAC

GdA

GSH

GPI

H

HRG

**Abbreviations**

[ <sup>3</sup> H]TdR	[methyl- <sup>3</sup> H]thymidine
aa	amino acid
aFGF	acidic fibroblast growth factor
ATIII	antithrombin III
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
BM	basement membrane
BSA	bovine serum albumin
BSS	balanced salt solution
cAMP	adenosine 3',5'-cyclic monophosphate
CHAPS	(3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate
cHRG	chicken histidine-rich glycoprotein
cpm	counts per minute
CR-heparin	carboxyl-reduced heparin
CSF-1	colony stimulating factor-1
DMSO	dimethylsulfoxide methyl sulfoxide
E9	embryonic day 9
E11	embryonic day 11
ECGF	endothelial cell growth factor
ECM	extracellular matrix
EDTA	ethylene diamine tetracetic acid
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FITC	fluorescein-5-isothiocyanate
FIU	fluorescence intensity unit
FN	fibronectin
FR1AP	mFR1 fused to human placental alkaline phosphatase
GAG	glycosaminoglycan
GalNAc	N-acetylgalactosamine
GlcA	glucuronic acid
GlcN	D-glucosamine
GPI	glycosyl phosphatidylinositol
h	human
hHRG	human histidine-rich glycoprotein

## Table of Contents

**Abbreviations cont.**

HRG	histidine-rich glycoprotein
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
HUVE	human umbilical vein endothelial
IdoA	iduronic acid
Ig	immunoglobulin
IL	interleukin
Kd	dissociation constant
mFR1	soluble form of murine FGF receptor 1
MW	molecular weight
ND	not determined
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PF4	platelet factor 4
PI-PLC	phosphatidylinositol-phospholipase C
PLC	phospholipase
PVC	polyvinylchloride
r	recombinant
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
t <sub>1/2</sub>	time required for half of the bound FGF to be released from the FGFR
TGF- $\beta$	transforming growth factor- $\beta$
TX-100	Triton X-100
VEGF	vascular endothelial growth factor



## Table of Contents

Statement .....	ii
Acknowledgements.....	iii
Abstract .....	iv
Abbreviations .....	ix
 <b>Chapter 1 Literature Review .....</b>	 <b>1</b>
1.1. Historical Note.....	1
1.2. The Heparin Binding Growth Factors.....	3
1.3. Introduction to the Fibroblast Growth Factor Family.....	3
1.4. Biological Functions of Acidic and Basic FGF.....	6
1.4.1. Cell Proliferation.....	7
1.4.2. Chemotaxis .....	8
1.4.3. Cell Spreading.....	8
1.4.4. Junctional Coupling.....	8
1.4.5. Differentiation .....	9
1.4.6. Angiogenesis.....	9
1.4.7. Wound Healing.....	10
1.4.8. Development .....	11
1.4.9. Reproductive Biology .....	12
1.4.10. Limb Regeneration.....	12
1.4.11. Modulation of the Endocrine Response .....	13
1.4.12. Effects of FGF on the Nervous System.....	13
1.4.13. Role of FGFs in Pathological Situations.....	14
1.4.13.1. FGFs as Oncogene Proteins.....	14
1.4.13.2. Presence of FGFs in Tumour Cells .....	14
1.4.13.3. FGFs and Tumour Angiogenesis.....	14
1.4.13.4. FGFs and Complications of Diabetes.....	15
1.4.13.5. Role of FGFs in Gastric Ulcers.....	15
1.5. General features of Acidic and Basic FGF.....	16
1.5.1. Gene Structure and FGF Gene Products.....	16
1.5.2. Binding Sites on FGFs .....	18
1.5.3. Three Dimensional Structure of FGFs .....	21
1.5.4. Tissue Distribution of FGFs.....	22
1.5.5. Secretion of FGF .....	23
1.6. FGF Receptors .....	24
1.6.1. Structural Features of FGF Receptors.....	26
1.6.2. FGF Receptor Sequence Homology .....	30
1.6.3. Tissue Distribution of FGF Receptors .....	31
1.6.4. FGFRs in Invertebrates.....	33
1.7. Heparin/HSPG Interaction with FGF .....	34
1.7.1. Structural Features of Heparin and Heparan Sulfate .....	34
1.7.2. Heparin Binding Domain of Acidic and Basic FGF .....	37
1.7.3. Nature of the Heparin/Heparan Sulfate Motif which Interacts with FGFs .....	39
1.7.4. Functional Relevance of Heparin/ Heparan Sulfate Binding by FGF .....	42
1.7.4.1. Secretion Model.....	43
1.7.4.2. Protection from Proteolysis Model.....	43



1.7.4.3. Reservoir Model .....	44
1.7.4.4. Receptor Binding Model .....	45
1.8. Role of HSPGs in FGF receptor binding .....	46
1.8.1. Conformational Change Model .....	46
1.8.2. Ternary Complex Model .....	46
1.8.3. Dimerization Model .....	47
1.9. Transmembrane and Intracellular Signalling by Acidic and Basic FGF .....	48
1.9.1. Receptor Dimerization .....	48
1.9.2. Intracellular Messengers Implicated in FGF Activity .....	49
1.10. Localization of FGF in the Nucleus .....	51
1.11. Aims of the Present Study .....	54
<b>Chapter 2 Materials and Methods .....</b>	<b>56</b>
2.1 Growth Factors and Heparin Binding Proteins .....	56
2.2. Polysaccharides .....	56
2.3. Fluoresceination and Iodination of Heparins .....	57
2.4. Iodination of cHRG .....	58
2.5. Effect of Heparins on Digestion of aFGF by Trypsin .....	58
2.6. Cell Culturing .....	59
2.7. Heparinase Treatment of 3T3 Cells .....	60
2.8. Isolation of HSPGs from 3T3 Fibroblasts .....	60
2.9. Binding of Radiolabeled Heparins to BALB/c 3T3 Cells .....	61
2.10. Binding of Radiolabeled Acidic and Basic FGF to BALB/c 3T3 Cells .....	62
2.11. Binding of Radiolabeled aFGF, bFGF and cHRG to Extracellular Matrices .....	64
2.12. Binding Affinity of Radiolabeled Heparin and CR-Heparin for Immobilized aFGF and bFGF .....	65
2.13. Rose Bengal Cell Adhesion Assay .....	66
2.14. Fluorescence Flow Cytometry .....	68
2.15. Soluble FGF Receptor Binding Assay .....	70
2.16. Effect of cHRG on Cell Attachment .....	71
2.17. Mitogenic assays .....	71
<b>Chapter 3 Acidic and Basic FGF Bind with Differing Affinity to the Same HSPG on BALB/c 3T3 Cells: Implications for Potentiation of Growth Factor Action by Heparin .....</b>	<b>74</b>
3.1. Introduction .....	74
3.2. Results .....	75
3.2.1. Binding of Acidic and Basic FGF to Cell Surface HSPGs .....	75
3.2.2. Role of Cell Surface HSPGs in Binding of FGFs to FGFR .....	77
3.2.3. Potentiation of Acidic and Basic FGF Action by Heparin .....	79
3.3. Discussion .....	80
3.4. Summary .....	86



<b>Chapter 4 Evidence that Carboxyl-Reduced Heparin Fails to Potentiate aFGF Activity via an Inability to Interact with Cell Surface Heparin Receptors.....</b>	<b>87</b>
4.1. Introduction .....	87
4.2. Results .....	88
4.2.1. General Properties of Carboxyl-Reduced Heparin.....	88
4.2.2. Potentiation of aFGF Activity by Heparin and CR-Heparin.....	89
4.2.3. Binding Affinities of Heparin and CR-Heparin for Acidic and Basic FGF .....	90
4.2.4. Protection of aFGF from Trypsin Digestion by Heparin.....	91
4.2.5. Binding of Radiolabeled aFGF to Heparinase Treated Cells.....	92
4.2.6. Binding of Heparin and CR-Heparin to Cell Surface Receptors.....	92
4.2.7. Effect of Heparin and CR-Heparin on the Binding of aFGF and bFGF to Soluble FGFR .....	93
4.3. Discussion.....	95
4.4. Summary.....	100
 <b>Chapter 5 Histidine-Rich Glycoprotein and Platelet Factor 4 Mask Heparan Sulfate Proteoglycans Recognized by Acidic and Basic Fibroblast Growth Factor.....</b>	 <b>102</b>
5.1. Introduction .....	102
5.2. Results .....	104
5.2.1. Binding of HRGs to Cell Surface HSPGs .....	104
5.2.2. Inhibition of Binding of Heparin Binding Proteins to Cell Surface HSPGs.....	106
5.2.3. Inhibition of Binding of Heparin Binding Proteins to HSPGs on the ECM .....	108
5.2.4. Inhibition of FGF Induced Mitogenesis by HRG .....	109
5.3. Discussion.....	111
5.4. Summary.....	115
 <b>Chapter 6. Final Discussion .....</b>	 <b>117</b>
6.1. Role of Cell Surface HSPGs in Binding of FGFs to the FGFR .....	117
6.2. Mode of Potentiation of aFGF Induced Mitogenic Activity by Heparin .....	120
6.3. Inhibition of Binding of FGFs to Cell Surface HSPGs by Heparin Binding Proteins .....	121
6.4. Future Work .....	122
 <b>References .....</b>	 <b>125</b>



## **Chapter 1**

### **Literature Review**

Recently, there has been a dramatic increase in the amount of research devoted to the Fibroblast Growth Factor family. This has meant that considerable progress has been made towards understanding their biological activities, physiological roles and clinical application. The Fibroblast Growth Factors, with their ubiquitous distribution, are now recognized as a family of growth factors whose members play a critical role in a wide range of normal and pathological processes. Yet there remains much to be understood about them, and in this introductory chapter I have attempted to outline the physiological processes in which FGFs are thought to be involved and to describe what is known to date, about their mechanism of action. After a brief introduction to the FGF family, I will concentrate on acidic and basic FGF for these are the two growth factors most pertinent to this thesis. Special emphasis has been placed on what is known about their interactions with cell surface receptors and, in particular, their interaction with heparan sulfate proteoglycans (HSPGs) and heparin. The relevance of these interactions to FGFs' mechanisms of action is also discussed. Recently a new nomenclature was introduced at a meeting of the New York Academy of Sciences which numbered the members of the Fibroblast Growth Factor Family as FGF-1 to FGF-7. However, for ease of identification, I have retained the old nomenclature of acidic and basic FGF throughout this thesis.

#### **1.1. Historical Note**

As early as 1939, Trowell et al.,(1939) and Hoffman (1940) detected mitogenic activity for fibroblasts in extracts of brain and then later in bovine pituitary (Armelin 1973; Gospodarowicz et al.,1974; Gospodarowicz 1975). It was not until the mid-1970's, that the polypeptide mitogen for 3T3 cells was purified from bovine pituitary (Gospodarowicz et al.,1975) and named fibroblast growth



factor and subsequently characterized as a basic polypeptide with mitogenic activity for mesoderm- and neuroectoderm-derived cells (Gospodarowicz 1983). However, high levels of contaminating myelin basic protein in the preparation hindered characterization of its structure (Westfall et al., 1978). Thomas and coworkers (1980) independently identified a polypeptide with an acidic isoelectric point from acid extracts of bovine brain and named it acidic fibroblast growth factor. Acidic FGF was mitogenic for endothelial cells (Lemmon et al., 1982) and on this criterion was initially distinguished from bFGF (Maciag et al., 1979; Lemmon et al., 1982; Thornton et al., 1983). In 1984, both aFGF (Thomas et al., 1984) and bFGF (Bohlen et al., 1984) were purified and characterized as potent heparin binding polypeptide mitogens for endothelial cells (Shing et al., 1984; D'Amore & Klagsbrun, 1984). Structural characterization of aFGF (Gimenez-Gallego et al., 1985) and bFGF (Baird et al., 1986) demonstrated their high amino acid sequence homology and these two growth factors formed the basis of a larger family of polypeptide growth factors.

In 1983, Thornton and her colleagues demonstrated that heparin could potentiate the biological activity of crude preparations of aFGF (Thornton et al., 1983). The discovery that aFGF (Maciag et al., 1984) and bFGF (Shing et al., 1984) have a strong binding affinity for heparin facilitated their purification by heparin affinity chromatography (Shing et al., 1983; Maciag et al., 1984). Heparin affinity chromatography is now an essential step in the characterization and purification of both aFGF and bFGF because aFGF elutes from immobilized heparin with 1.0M NaCl whereas bFGF requires at least 1.6M NaCl for elution (Shing et al., 1984). Over twenty growth factors that had been previously isolated from various tissues and given different names were able to be identified as either aFGF or bFGF by heparin affinity chromatography.



## **1.2. The Heparin Binding Growth Factors**

The number of cytokines and growth factors which have been shown to bind heparin and HSPGs is rapidly increasing (Table 1.1). Although the members of the heparin binding growth factor family, described to date, appear to exhibit lower affinities for heparin than do the FGFs, the reasons proposed to explain the functional significance of such an interaction are similar to those put forward to explain FGF binding to HSPGs. Heparin-binding cytokines are thought to bind to HSPGs on the cell surface and in the extracellular matrix (ECM), enabling their retention near their site of secretion and facilitating the regulation and differentiation events of hematopoiesis (Ramsden & Rider 1992; Gordon et al., 1987; Roberts et al., 1988; Keating & Gordon 1988). Interleukin-3 and granulocyte macrophage-colony stimulating factor have been shown to stimulate receptors on colony-forming cells whilst bound to HSPGs synthesized by stromal cells (Roberts et al., 1988). Heparin enhances the binding of vascular endothelial cell growth factor (VEGF) to VEGF receptors and it seems likely that VEGF may be required to interact with cell-surface HSPGs before it can interact with VEGF receptors (Gitay-Goren et al., 1992). While bound to HSPG, transforming growth factor- $\beta$  is held at the cell surface and protected from  $\alpha$ 2-microglobulin. Pleiotrophin is also thought to be sequestered by HSPGs in the ECM. Clearly the full significance of HSPGs in the modulation of cytokine and growth factor activity is yet to be realised.

## **1.3. Introduction to the Fibroblast Growth Factor Family**

Acidic and basic FGF (termed FGF-1 and FGF-2) are ubiquitously distributed throughout the body and have been purified from organs such as the adrenal gland (Gospodarowicz et al., 1986a), bone (Hauschka et al., 1986) cartilage (Sullivan & Klagsbrun 1985), corpus luteum (Gospodarowicz et al., 1985a) hypothalamus (Klagsbrun & Shing 1985), kidney (Baird et al., 1985a), liver (Ueno et al., 1986), placenta (Gospodarowicz et al., 1985b), prostate (Nishi et al., 1985), retina (Baird et al., 1985b), testis (Ueno et al., 1987) and thymus



Table 1.1: Heparin Binding Growth Factors

Common name	Acronym	Reference
acidic fibroblast growth factor	FGF1	Burgess & Maciag (1989)
basic fibroblast growth factor	FGF2	Burgess & Maciag (1989)
int-2	FGF3	Yoshida et al.,(1988)
Kaposi sarcoma FGF	FGF4	Yoshida et al.,(1988)
fibroblast growth factor-5	FGF5	Nguyen et al.,(1988)
fibroblast growth factor-6	FGF6	Nguyen et al.,(1988)
keratinocyte growth factor	FGF7	Rubin et al.,(1989)
androgen-induced growth factor	FGF8	Tanaka et al.,(1992)
glia-activating factor	FGF9	Miyamoto et al.,(1993)
interleukin 1 $\alpha$	IL-1 $\alpha$	Ramsden & Rider (1992)
interleukin 1 $\beta$	IL-1 $\beta$	Ramsden & Rider (1992)
interleukin 2	IL-2	Ramsden & Rider (1992)
interleukin 3	IL-3	Roberts et al.,(1988)
interleukin 6	IL-6	Ramsden & Rider (1992)
granulocyte & macrophage colony stimulating factor	GM-CSF	Keating & Gordon (1988), Gordon et al.,(1987)
heparin binding epidermal growth factor	HB-EGF	Higashiyama et al (1990)
transforming growth factor $\beta$	TGF- $\beta$	McCaffrey & Falcone (1992)
vascular endothelial growth factor	VEGF	Gitay-Goren et al.,(1992)
platelet-derived growth factor	PDGF	Fager et al.,(1992)
pleiotrophin	-	Milner et al.,(1989)
midkine	MK	Tomomura et al.,(1990)



Table 1.1. continued:

Common name	Acronym	Reference
hepatocyte growth factor	HGF	Nakamura et al.,(1986)
insulin-like growth factor I	IGF-1	Myers et al.,(1993)
insulin-like growth factor 2	IGF-2	Myers et al.,(1993)
amphiregulin	-	Myers et al.,(1993)
Schwannoma-derived growth factor	-	Klagsbrun & Baird (1991)

(Gospodarowicz et al., 1986b). Highly purified preparations of aFGF and bFGF were subjected to amino acid sequencing and cDNAs for aFGF (Gimenez-Gallego et al., 1985; Thomas et al., 1985; Jaye et al., 1986) and bFGF (Esch et al., 1985; Abraham et al., 1986a,b,c; Kurokawa et al., 1987) have been cloned.

Seven additional members of the FGF family have been identified to date, on the basis of amino acid sequence homologies (Tables 1.2 & 1.3). These proteins are the product of the int-2 oncogene (FGF3) (Moore et al., 1986); the product of the hst oncogene (Kaposi sarcoma FGF or FGF-4) (Taira et al., 1987; Bovi & Basilico 1987); FGF-5 (Zhan et al., 1988); FGF-6 (Marics et al., 1989); keratinocyte growth factor (KGF or FGF7; Finch et al., 1989; Rubin et al., 1989); androgen-induced growth factor (AIGF or FGF-8) and glia-activating factor (GAF or FGF-9). Int-2, hst/KFGF, FGF-5, FGF-6, KGF, AIGF and GAF exhibit 30-45% sequence homology with aFGF and bFGF and share with aFGF and bFGF the ability to bind heparin. In contrast to aFGF, bFGF and GAF, the other family members contain signal peptide sequences encoded by their mRNA transcripts.

The int-2 (FGF-3) gene, a proto-oncogene, was discovered through its transcriptional activation by mouse mammary tumour virus (Dickson et al., 1984; Peters et al., 1983) and its ability to participate in mammary tumorigenesis. It seems that it only functions in specialized cell types such as in developing embryos at a variety of stages (Jakobovits et al., 1986; Wilkinson et al., 1989) and may be a cell-to-cell signaling molecule (Dickson & Peters 1987).

The FGF-4 gene, previously known as hst-1 or KFGF, a transforming gene, was the second oncogene found to encode a growth factor. It is activated in DNAs of a variety of tissues, for example, noncancerous gastrointestinal mucosae. The gene is 35kbp downstream from the int-2 gene and in the same



Table 1.2. (cont.) General Features of Fibroblast Growth Factors

Common name	Generally accepted Acronyms	Proposed name	Gene on human chromosome	Primary translation product (aa)	Signal sequence present	[NaCl] for elution from heparin	FGF Receptor	% Sequence identity to bFGF	Oncogenic potential
Karposi Sarcoma FGF	ksFGF/ K-FGF, hst-1/ HSTF-1	FGF-4	11q13	206	yes	1.2-1.3M	ND	42	yes
Fibroblast Growth Factor-5	hst-1 FGF-5 HSTF1	FGF-5	4q21	267	yes	1.0-1.5M	FGFR1 FGFR2	43	yes
Fibroblast Growth Factor-6 Hst-1 related gene	FGF-6 hst-2	FGF-6	12q13	198 (murine)	yes	ND			yes
Keratinocyte Growth Factor	KGF	FGF-7		194	yes	0.6M	FGFR2 (variant)	28	ND
Androgen-Induced Growth Factor	AIGF	FGF-8	ND	215	yes	2.0M	ND	30	yes?
Glia-Activating Factor	GAF	FGF-9	ND	208	no	0.8M	ND	30	yes?

Information for Table gathered from Olwin et al.,(1991); Klagsbrun & Baird (1991); Tanaka et al.,(1992); Miyamoto et al.,(1993)

Table 1.2: General Features of the Fibroblast Growth Factors

Common name	Generally accepted acronyms	Proposed name	Gene on human chromosome	Primary translation product (aa)	Signal Sequence present	[NaCl] for elution from heparin	FGF Receptor	% Sequence identity to bFGF	Oncogenic potential
Acidic Fibroblast Growth Factor, Endothelial Cell Growth Factor, Heparin-Binding Growth Factor-1,	aFGF Acidic FGF, ECGF, HBGF-1	FGF-1	5q31-33	154	no	1.0 M	FGFR1 FGFR2 FGFR3 FGFR4	53	no
Basic Fibroblast Growth Factor, Heparin-Binding Growth Factor	bFGF, Basic FGF, HBGF-2	FGF-2	4q25	154 196 201 210	no	1.6M	FGFR1 FGFR2 FGFR3	100	no
int-2	int-2	FGF-3	11q13	239 271	yes	ND	ND	42	yes

Table 1.2. (cont.) General Features of Fibroblast Growth Factors

Common name	Generally accepted Acronyms	Proposed name	Gene on human chromosome	Primary translation product (aa)	Signal sequence present	[NaCl] for elution from heparin	FGF Receptor	% Sequence identity to bFGF	Oncogenic potential
Karposi Sarcoma FGF	ksFGF/ K-FGF, hst-1/ HSTF-1	FGF-4	11q13	206	yes	1.2-1.3M	ND	42	yes
Fibroblast Growth Factor-5	hst-1 FGF-5 HSTF1	FGF-5	4q21	267	yes	1.0-1.5M	FGFR1 FGFR2	43	yes
Fibroblast Growth Factor-6 Hst-1 related gene	FGF-6 hst-2	FGF-6	12q13	198 (murine)	yes	ND			yes
Keratinocyte Growth Factor	KGF	FGF-7		194	yes	0.6M	FGFR2 (variant)	28	ND
Androgen-Induced Growth Factor	AIGF	FGF-8	ND	215	yes	2.0M	ND	30	yes?
Glia-Activating Factor	GAF	FGF-9	ND	208	no	0.8M	ND	30	yes?

Information for Table gathered from Olwin et al.,(1991); Klagsbrun & Baird (1991); Tanaka et al.,(1992); Miyamoto et al.,(1993)



Table 1.3: Tissue Distribution of FGFs

Growth Factor	Tissue	Reference
FGF-1	neural, kidney, prostate, cardiac muscle	Casscells et al., (1990), Crabb et al.,(1986), D'Amore & Klagsbrun (1984), Thomas et al.,(1984)
FGF-2	brain, pituitary, retina, adrenal, corpus luteum, testes, skeletal, cardiac, some blood vessels, in all organs examined	Esch et al.,(1985), Baird et al.,(1985b, 1986), Bohlen et al., (1985),Gimenez- Gallego et al.,(1986), Gospodarowicz et al., (1984, 1985a,1986a) Ueno et al.,(1987), Gonzalez et al.,(1990) Joseph-Silverstein et al.,(1989)
FGF-3	embryo, foetus, cerebellum, sensory region of inner ear, retina	Jakobovitis et al., (1986), Wilkinson et al.,(1989)
FGF-4	very early embryo, foetus	Herbert et al.,(1990)
FGF-5	most phases of embryonic & foetal growth, adult brain, specific types of mesenchyme & striated muscle	Haub & Goldfarb (1991)
FGF-6	embryo, adult skeletal muscle & heart	Delli Bovi et al., (1988), Kimelman et al.,(1988)
FGF-7	adult kidney, colon, ilium, epithelial cells	Finch et al.,(1989) Rubin et al.,(1989)
FGF-8	tumour cells	Tanaka et al.,(1992)
FGF-9	brain, kidney	Miyamoto et al.,(1993)

transcriptional orientation (Wada et al. 1988). Both genes are coamplified in several types of human cancers (Yoshida et al., 1988; Tsuda et al., 1989; Adnane et al., 1989). The FGF-4 gene is expressed in the apical ectodermal ridge of the early mouse limb-bud and the secreted protein, FGF-4, stimulates the proliferation of the underlying mesenchyme (Niswander & Martin 1992; Suzuki et al., 1992). Therefore, FGF-4 is thought to cause limb outgrowth by stimulating the proliferation of cells in distal limb mesenchyme (Niswander & Martin 1993).

FGF-5 is thought to mediate a diverse set of events during embryogenesis. Although expressed through most phases of embryogenesis (Herbert et al., 1990), FGF-5 gene expression is temporally restricted and tissue specific. It is also expressed weakly and exclusively in the adult central nervous system (Haub et al., 1990).

The restricted pattern of FGF-6 gene expression suggests a specific activity of FGF-6 in the embryo and in cardiac and skeletal muscle cells. The role of FGF-6 in muscle formation is not known. FGF-6 has a transforming capacity comparable to, and sequence similarities, with FGF-4 (hst/KFGF) (Sakamoto et al., 1988; Miyagawa et al., 1988; Delli-Bovi et al., 1988). Despite this intrinsic transforming capacity, the FGF-6 gene has not been detected in transfection assays (Sakamoto et al., 1986; Bovi & Basilico 1987) and has never been found altered in human tumours, suggesting that the intrinsic oncogenic capacity of the FGF genes must be repressed *in vivo*.

Keratinocyte growth factor (FGF-7) acts specifically as a paracrine modulator of epithelial cell growth (Finch et al., 1989). It has no mitogenic activity on fibroblasts, endothelial cells, melanocytes or other non-epithelial targets of FGF action. KGF receptors bind aFGF with a high affinity but have a much lower affinity for bFGF.



Androgen-induced growth factor (FGF-8) is possibly the first hormone-induced autocrine growth factor identified. It has remarkable stimulatory effects on a mouse mammary carcinoma cell line (SC-3 cells) and is secreted by these cells in response to androgen, stimulating them in an autocrine manner (Tanaka et al., 1992).

Glia-activating factor promotes glial cell proliferation (Naruo et al., 1993) and is therefore implicated in the development and maintenance of cells of the central nervous system. Besides being expressed in the brain, its gene is also expressed in kidney, suggesting that this growth factor may also act on other organs in an endocrine manner. It consists of a single peptide and there are three molecular weight isoforms of 30, 29 and 25kDa, each having an N-linked sugar chain of 3kDa. In addition to promoting the proliferation of rat glial cells, it also activates O-2A progenitor cells, PC-12 cells, BALB/c 3T3 cells but not human umbilical vein endothelial cells (Naruo et al., 1993). When transfected into BALB/c 3T3 cells it exhibits transforming potency and therefore may be involved in oncogenesis of NMC-G1 cells. Although lacking a typical signal sequence, it is clearly secreted from cells and it is proposed that it may have a functional signal sequence in the N-terminal domain which is uncleavable in the secretion step (Miyamoto et al., 1993).

I will now concentrate on acidic and basic FGF because these are the two most relevant to this thesis.

#### **1.4. Biological Functions of Acidic and Basic FGF**

FGFs are important in proliferation, differentiation and chemotaxis of various cell types in culture. These effects *in vitro* implicate them in the processes of angiogenesis which involves endothelial cell proliferation, migration and



differentiation, in wound healing which requires migration and proliferation of fibroblasts, in nerve regeneration, in cartilage repair and in embryogenesis.

#### 1.4.1. Cell Proliferation

Both aFGF and bFGF are potent mitogens *in vitro*, being active in the 0.1 to 1.0 ng/ml range, although bFGF is often 10-30 fold more active than aFGF (Gospodarowicz 1987). It appears that cells derived from the three embryonic germ layers (mesoderm, ectoderm and endoderm) respond to FGF (reviewed in Gospodarowicz 1990). Cells which are FGF-sensitive include endothelial cells (Folkman & Klagsbrun 1987; Gospodarowicz et al., 1978; Maciag et al., 1981), adrenal cortex cells (Schweigerer et al., 1987a; Gospodarowicz et al., 1977), prostatic cells (McKeehan et al., 1984; Hoshi & McKeehan 1984; Chaproniere & McKeehan 1986), retinal epithelial cells (Arruti & Courtois 1978; Barritault et al., 1981; Tarsio et al., 1983; Courty et al., 1985; Baird et al., 1985b; Courty et al., 1986;), oligodendrocytes (Eccleston & Silberberg 1985) astrocytes (Wu et al., 1988), chondrocytes (Kato & Takayama 1984; Kato et al., 1987; Kato & Gospodarowicz 1985), myoblasts (Kardami et al., 1985; Hauschka et al., 1986; Eccleston & Silberberg 1985; Wice et al., 1987) and osteoblasts (Canalis et al., 1987; Rodan et al., 1987; Canalis & Lian 1988). Human melanocytes respond to the mitogenic influence of bFGF but not aFGF (Halaban et al., 1987), and most avian and mammalian cell types respond to both polypeptides (reviewed by Lobb 1988a). Acidic and basic FGF are also potent mitogens for a number of established cell lines including BALB/c and Swiss 3T3 cells, LElI cells, BHK21 cells, CHO cells and rhabdomyosarcoma cells (reviewed by Thomas & Gimenez-Gallego 1986; Gospodarowicz et al., 1986b). In many cases, the mitogenic effect of FGFs on normal diploid mammalian cells significantly delays their premature senescence *in vitro* (Maciag et al., 1981; Gospodarowicz & Bialecki 1978) by inducing early G<sub>0</sub> to G<sub>1</sub> cell cycle events (Wice et al., 1987; Gospodarowicz 1983; Maciag 1984) and cellular competence (Stiles et al., 1979).



#### **1.4.2. Chemotaxis**

Acidic FGF is chemotactic for endothelial cells, fibroblasts and astroglial cells (Terranova et al., 1985; Senior et al., 1986). It is not known whether the chemotactic activities of FGFs are mediated through the same receptor systems involved in the induction of cell proliferation but there is evidence to suggest that chemotactic and mitogenic activities are distinct. Linemeyer et al., (1987) have shown that cell division and cell migration in response to aFGF require mutually exclusive cytoskeletal arrangements and that high concentrations of a raFGF(15-154) fragment which inhibited thymidine incorporation into BALB/c 3T3 cells stimulated chemokinesis and chemotaxis of these cells. It is proposed that high concentrations of aFGF could stimulate migration of endothelial cells or fibroblasts to the site of a wound or tumour and then diffusion mediated decreases in the concentration of growth factors would result in stimulation of proliferation of the "recruited" cells (Burgess & Maciag 1989).

#### **1.4.3. Cell Spreading**

In 1978, Folkman and Moscona, demonstrated the importance of cell spreading in cell proliferation by showing that DNA synthesis induced by serum correlated with the degree of cell spreading. More recently, Ingber (1990a), has shown that the mitogenic effect of bFGF depends on cell spreading which may in turn be modulated by proteins in the ECM (Elliot et al., 1992).

#### **1.4.4. Junctional Coupling**

Basic FGF also induces junctional coupling between endothelial cells (Pepper & Meda 1992), increasing junctional communication and connexin 43 expression in microvascular endothelial cells. Adjacent endothelial cells in the vascular endothelium are linked by intercellular tight junctions and gap junctions. Gap junctions are transmembranous channels which regulate the passage of small molecules and ions between adjacent cells (reviewed by



Bennett et al.,1991). A bFGF-induced increase in coupling may represent a physiological mechanism which allows cells on the leading front of a wound's edge to communicate with adjacent cells and cells located further back. Endothelial cell coupling may also play a role in arteriolar vasodilation (Segal & Duling 1986, 1987, 1989) and the maintenance of intercellular contacts is probably important in the formation of structurally and functionally competent capillary blood vessels during angiogenesis.

#### **1.4.5. Differentiation**

Both aFGF and bFGF promote the cellular differentiation and survival *in vitro* of a variety of cells that migrate from the neural crest, including hippocampal and cortex-derived cells *in vitro* (Eccleston & Silberberg 1985; Neufeld et al.,1987; Walicke et al.,1986; Unsicker et al.,1987; Lipton et al.,1988; Schubert et al.,1987; Wu et al.,1988). Basic FGF induces adipocyte differentiation *in vitro* (Serrero & Khoo 1982) and both growth factors promote neurite extension in PC12 cells, a rat pheochromocytoma cell line (Togari et al.,1983,1985). Both FGFs also appear to have a role in both mesoderm differentiation and mesoderm patterning throughout gastrulation (Amaya et al.,1991).

#### **1.4.6. Angiogenesis**

Angiogenesis is the process by which new blood vessels are formed (Folkman 1991) and is essential in reproduction, development and wound repair (Folkman & Shing 1992). In these processes, angiogenesis is highly regulated, although, many diseases are driven by unregulated angiogenesis. Currently, there are eight polypeptides known to be angiogenic, two of which are aFGF and bFGF. Basic FGF is angiogenic when injected extravascularly (Whalen et al.,1989). The biological activities of aFGF and bFGF which implicate them in angiogenesis are:

- 1) their mitogenicity for a wide range of cell types. Basic FGF has been shown to be a potent angiogenic factor *in vivo* when tested in the rabbit cornea, chick chorioallantoic membrane and hamster cheek pouch assays (Gospodarowicz



et al.,1979; 1984; 1985a; 1986a,b). Acidic FGF, although less potent than bFGF, is also angiogenic in the chorioallantoic membrane and rabbit cornea assays (Thomas et al.,1985; Lobb et al.,1985). Furthermore, vasodilation of the parent venule occurs before the emergence of the first capillary sprout and it is thought that this may stretch the endothelial cells making them responsive to bFGF or other growth factors (Ingber 1990b).

2) their ability to bind HSPGs (Vlodavsky et al.,1987a). Basic FGF stored in the ECM, bound to HSPGs, may be mobilized by collagenases or heparanases which have been secreted by endothelial cells or other vessel associated cells.

3) their ability to bind copper (Shing 1988; Baird & Ling 1987). Certain copper complexes, eg, copper/heparin are angiogenic.

4) their ability to stimulate endothelial cells to migrate and form tubes (Montesano et al.,1986)

5) their ability to increase production of proteases and plasminogen activator (Moscatelli et al.,1986; Presta et al.,1986)

#### **1.4.7. Wound Healing**

Acidic FGF has been shown to promote repair of experimentally damaged rat arterial endothelium and to inhibit the accompanying pathological intimal thickening (Bjornsson et al.,1991) . After intravascular injury, a thickening of the intimal smooth muscle layer adjacent to the lumen and a subsequent decrease in the vascular lumen diameter occurs when smooth muscle cells migrate and proliferate into the intima and lay down ECM. Although the mechanism by which aFGF inhibits intimal thickening is unknown it is suggested that circulating aFGF has direct mitogenic and chemokinetic stimulatory effects on vascular endothelial cells. It is proposed that repair of damaged endothelium might inhibit intimal thickening by limiting the exposure of the underlying smooth muscle cell to stimulatory factors (Bjornsson et al.,1991). Such intimal thickening occurs in humans following balloon



angioplasty and is a major cause of coronary artery restenosis. Acidic FGF could be used therapeutically in such situations.

The interaction between ECM and growth factors has been shown to be an integral component of the wound healing process. It appears that the ECM does far more than stabilize the fibrin clot via interaction with fibronectin (FN). Soon after injury, the matrix releases bFGF and signals cells to begin manufacturing bFGF and expressing FGF receptors on their cell surfaces (Baird 1991 Keystone conference). Basic FGF and the other growth factors released, cause a variety of cell types to proliferate but the matrix immobilizes the growth factors, preventing them from diffusing out of the wounded area and therefore ensuring only a local response. Chemical signals guide cells toward the wounded area. Growth factors enable keratinocytes to migrate through the ECM to get to the wound bed, by stimulating the cells to produce collagenase (David Woodley, Keystone conference 1991). Fibroblasts also migrate to the wound site where their response to FGF is also controlled by the ECM (Grinnell 1991 Keystone conference). The end of the wound healing process requires that cell proliferation is turned off (Grinnell, Keystone conference 1991). In response to TGF- $\beta$ , secreted by inflammatory cells, the fibroblasts make collagen to restore the damaged matrix, consequently, FGFs are sequestered by the ECM and it is proposed that cells cease to proliferate as a result of the removal of the FGF stimulus (Skerrett 1991).

#### **1.4.8. Development**

The ability of FGFs to promote proliferation of cells derived from each of the germ layers, and to influence the differentiation of a variety of cell types, combined with the detection of bFGF in *Xenopus* eggs (Slack et al., 1987; Knochel et al., 1987; Grunz et al., 1988), unfertilized chicken eggs and the chick embryo (Seed et al., 1988) suggests a major role for both factors during development. In fact, there is experimental evidence to suggest that they may



play a major role during the development of the nervous, skeletal, and vascular systems (reviewed by Gospodarowicz et al., 1987a). By expressing a dominant negative mutant form of the FGF receptor in frog oocytes, Amaya et al., (1991), have demonstrated the importance of the FGF signaling pathway in embryogenesis, ie., its involvement in the early establishment of the posterior and lateral structures of the frog embryo. It is yet to be determined which regions of the embryo are involved in the FGF signaling pathway.

#### **1.4.9. Reproductive Biology**

Basic FGF has been shown to be a potent inhibitor of aromatase activity in cultured granulosa cells and peripheral fibroblasts (Baird & Walicke 1989) and also of testosterone synthesis *in vitro*. It is also present in the corpus luteum and may be associated with the neovascular response that underlies normal menses, implantation and fetal development, ie., it could be an ovarian angiogenic factor. Basic FGF may well be involved in some types of ovarian dysfunction many of which can be traced to problems of vascularization (Baird & Walicke 1989).

#### **1.4.10. Limb Regeneration**

Some amphibian species are able to regenerate lost appendages and there is a strong possibility that bFGF is involved in one of the earlier steps of amphibian limb regeneration, namely, recruitment of primitive cells leading to blastema formation. A neurotrophic effect is necessary for cell proliferation involved in blastema formation but not for later events such as differentiation of blastema cells into muscle cartilage and other tissues of the new limb (Powell et al., 1969; Singer 1974). Basic FGF is a potent mitogen for myoblasts and chondrocytes (Gospodarowicz & Mescher 1977), the two cell types from which blastema cells are thought to originate (Hay 1974; Gospodarowicz et al., 1976). Infused FGF has also been shown to promote the resumption of mitotic activity in denervated newt limb blastemas (Mescher & Gospodarowicz 1979). Basic FGF has also been demonstrated to maintain total acetylcholinesterase activity



in cultured newt triceps muscle above that in untreated controls, (Carlone et al., 1981), and to promote heterotrophic growth of adult frog (*Rana pipiens*) forelimbs after amputation (Gospodarowicz et al., 1975).

#### **1.4.11. Modulation of the Endocrine Response**

The anterior pituitary gland contains the highest concentration of bFGF in the body (Gospodarowicz et al., 1986c). It has been suggested that bFGF is required in the pituitary to promote angiogenesis (Ferrara et al., 1987), to stimulate cell growth (Hayashi et al., 1978) and hormonal secretion (Baird et al., 1985c), and to inhibit DNA synthesis (Black et al., 1990). However, these ascribed functions are controversial and the results of Inoue et al., (1991) suggest that bFGF in the pituitary may stimulate pituitary glandular cell adhesion to the ECM which may then allow inhibitory control of cell proliferation by bFGF.

#### **1.4.12. Effects of FGF on the Nervous System**

In tissue culture, bFGF has trophic effects on neurons (Walicke 1989), is a mitogen for neuroblasts from young embryos and enhances the survival of post mitotic differentiated neurons. A neurotrophic function has also been suggested for aFGF. Acidic FGF promotes neuronal differentiation and neurite outgrowth in cultures of rat PC12 cells (Rydel & Greene 1987), increases neurite initiation and elongation in cultured rat retinal ganglion cells (Lipton et al., 1988), promotes neuronal survival in fetal rat hippocampal and fetal chick spinal cord cultures (Unsicker et al., 1987; Walicke & Baird 1988) and promotes neuronal survival in fetal rat hippocampal and fetal chick spinal cord cultures (Unsicker et al., 1987; Walicke & Baird, 1988) as well as in cultures of fetal rat and chick peripheral ganglia (Unsicker et al., 1987). Although bFGF is generally more potent than aFGF *in vitro*, retinal ganglion cells appear to specifically require aFGF. Both FGFs enhance the growth of neuritic processes implicating them in regulation of brain growth. Not only are neurons FGF-responsive but astrocytes and oligodendrocytes are also. Research is



being carried out to assess the usefulness of bFGF in improving neuronal recovery after brain lesions. Infusion of bFGF to the severed sciatic nerve enhances the rate of nerve regeneration (Cuevas et al., 1988) and bFGF administered to the stump of the severed optic nerve can increase the survival of retinal ganglion cells (Cuevas et al., 1988; Sievers et al., 1987).

#### **1.4.13. Role of FGFs in Pathological Situations**

##### **1.4.13.1. FGFs as Oncogene Proteins**

Several groups have shown that overexpression of FGF by cells can lead to transformation which suggests that FGFs can act as proto-oncogenes (Rogelj et al., 1988; Jaye et al., 1988). Each of the FGF-related oncogenes, *hst*, *int-2* and *FGF-5* are associated with cell transformation. The steps that lead to overexpression of FGFs and FGF-dependent tumour growth are yet to be defined.

##### **1.4.13.2. Presence of FGFs in Tumour Cells**

FGFs have also been isolated from cultured tumour cells and tumour tissue (reviewed by Gospodarowicz et al., 1986c). They are proposed to stimulate tumour cell growth as autocrine growth factors and also as tumour angiogenic factors (Gospodarowicz et al., 1986c; Schweigerer et al., 1987b; Folkman & Klagsbrun 1987; Burgess & Maciag 1989; Nagao & Nishikawa 1989). Basic FGF has been found in rat Rhodamine fibrosarcomal tissue, (Nagao & Nishikawa 1989), human epidermal carcinoma cells (Masuda et al., 1987) and human cholangiocellular carcinoma cells (Matsuzaki et al., 1990). Acidic FGF has also been found in tumours, for example, a human neuroblastoma cell line (Huang et al., 1987) and in human brain tumours (Takahashi et al., 1990).

##### **1.4.13.3. FGFs and Tumour Angiogenesis**

FGFs may also promote solid tumour growth by vascularizing the invading tissue (Folkman & Klagsbrun 1987). The importance of angiogenesis in the development of solid tumours has been established by Folkman and



colleagues, over the last 20 years. In many cases, FGFs are produced by tumour cells and may be a component of the activity attributed to tumour angiogenesis factor or alternatively, the tumour may rely on the "host cells' FGF" to vascularize the tumour. In addition, tumours may recruit mast cells loaded with heparin and upon degranulation heparin may bind FGF (produced by tumour cells) and amplify its response. It is therefore extremely important to understand the regulation of endogenous FGF synthesis with a view to preventing FGFs from being mobilized by the invading tumour cells and inducing vascularization of the tumour. For example, in arthritis, new capillary blood vessels invade the joint and destroy cartilage and ocular neovascularization is the most common cause of blindness.

#### **1.4.13.4. FGFs and Complications of Diabetes**

There is strong evidence to suggest that some of the complications of diabetes are mediated by FGFs. FGFs are angiogenic and many of the complications associated with diabetes are associated with microvascular pathology in the eye, kidney and peripheral nerves (Baird & Walicke 1989). For example, new capillaries in the retina invade the vitreous, bleed and cause blindness (Folkman 1987). Furthermore, each of the elements which modify endogenous FGF availability, that is, carbohydrates, glycosaminoglycans (GAGs) and ECM are also modified in diabetes (Baird & Walicke 1989). Clinical trials are underway to assess the effects of aFGF, bFGF, PDGF and TGF- $\beta$  on patients with non-healing dermal ulcers resulting from diabetes.

#### **1.4.13.5. Role of FGFs in Gastric Ulcers**

There has been much experimental evidence in support of a role for FGF in the healing of gastrointestinal ulcers. The presence of aFGF and the FGF receptor, flg, at the wound margin of acetic-acid-induced gastric ulcers in rats has been demonstrated (Hansson & Norstrom 1991). Oral treatment with an acid-resistant mutein bFGF (TGF-580) has been shown to accelerate the



healing of both acetic-acid-induced gastric ulcers (Sato et al., 1991) and cysteamine-induced duodenal ulcers in rats (Folkman et al., 1990; Fitzpatrick et al., 1992). Folkman et al., (1991), have shown that endogenous bFGF exists in the rat and human gastric and duodenal mucosa but is sensitive to acid degradation. Sucralfate and sucrose octasulfate both bind bFGF with a high affinity and are able to protect bFGF from acid degradation. Consequently, the oral administration of sucralfate elevates local levels of bFGF in the ulcer bed, enabling bFGF to accelerate ulcer healing by stimulating angiogenesis. Folkman et al., (1991) propose that gastric acid encourages ulcer formation by degrading and inactivating endogenous bFGF and that administration of an acid-resistant form of bFGF (exogenous) could provide a form of replacement therapy.

### **1.5. General features of Acidic and Basic FGF**

Some of the general features of acidic and basic FGF will be discussed in this section and are summarized in Table 1.4.

#### **1.5.1. Gene Structure and FGF Gene Products**

The high degree of sequence homology, (55%), between bFGF and aFGF suggests that they are derived from a single ancestral gene and that they have become separate gene products through a process of gene duplication and evolutionary divergence (Abraham et al., 1986c). Acidic and basic FGF are encoded by separate genes, each present as a single copy (Jaye et al., 1986; Abraham et al., 1986a,b,c). The gene for aFGF is found on human chromosome 5, between bands 5q 31.3 and 5q 33.3 (Jaye et al., 1986) whereas the gene for basic FGF is present on chromosome 4, at 4q25 (Mergia et al., 1986). The genes for epidermal growth factor and interleukin-2 have also been mapped to chromosome 4 (McKusick 1985), while the genes for c-fms (the receptor for colony stimulating factor-1) and human PDGF receptor are localized on the short arm of chromosome 5 (Jaye et al., 1986).

Table 1.4: Biochemical Properties of Acidic and Basic FGF

Properties	Acidic FGF	Basic FGF
Target cells	endothelial , epithelial, mesenchyme & neuronal cell types	
Molecular weight (kDa)	16	18,22,23,24
Number of amino acids	154	154,196,201,210
	55% sequence homology	
Isoelectric point	5.6	9.6
Signal sequence for secretion	absent	absent
Human chromosome with FGF gene	5	4
FGF receptor	FGFR1,FGFR2 FGFR3, FGFR4	FGFR1,FGFR2 FGFR3
FGFR sites/cell	$5 \times 10^3$ - $5 \times 10^4$	$3 \times 10^3$ - $8 \times 10^4$
HSPG binding sites/cell	$0.5 \times 10^6$ - $2 \times 10^6$	$0.5 \times 10^6$ - $2 \times 10^6$
Kd for HSPG (nM)	2-10	2
Elution from heparin	1M NaCl	1.6M NaCl
Potentiation of mitogenic activity by heparin	yes	no
3D structure	similar: both have 12 antiparallel $\beta$ strands with 3-fold internal symmetry	
Melting temperature	42°C	57°C
Effect of copper	reduced mitogenesis & heparin binding	unaffected
Effect of thrombin	degraded & inactivated	unaffected

Based on reviews by D'Amore (1990b); Gospodarowicz et al.,(1986b); Rifkin & Moscatelli (1989); Lobb (1988b); Burgess & Maciag (1989).



Both aFGF and bFGF genes contain three exons separated by two large introns at similar locations (Mergia et al., 1986; Abraham et al., 1986a,b). Comparison of the amino acid sequences of the different FGF family members has shown that FGFs tend to have sequence similarities in a "core" portion coded for by the 3' half of exon 1, exon 2 and exon 3, but differ greatly in the portion coded for by the 5' half of exon 1. The gene for bFGF is at least 36kb long (Abraham et al., 1986a,b,c), the two introns each being 16kb long. Genomic sequences 5' of exon 1 contain five GC boxes, rather than the typical TATA or CAAT boxes, and are thought to represent SP-1 binding sites and one potential nuclear transcription factor, AP-1, binding site (Florkiewicz et al., 1991a). The data of Florkiewicz et al., (1991a) suggest that the human bFGF gene promoter contains two negative regulatory regions. The second of these regions shares some homology with a negative regulatory sequence motif found in the c-myc gene promoter (Hay et al., 1989). It is yet to be determined whether bFGF DNA sequence domains with negative regulatory properties act via a factor-dependent or factor-independent mechanism. However, the similarity between myc and bFGF in this region raises the possibility that fos or fos-like proteins may be a part of the protein(s) complex that mediates regulatory activity (Florkiewicz et al., 1991a). It is not yet known whether similar regulatory domains exist within the promoter region of other members of the FGF family.

Following transcription, a single bFGF mRNA transcript is translated into a set of four coexpressed isoforms with apparent molecular weights of 24, 23, 22 and 18kDa (Florkiewicz & Sommer 1989; Prats et al., 1989). In contrast, evidence suggests that the various truncated forms of aFGF are artifacts of the purification procedures (McKeehan & Crabb 1987). The translation of the three larger molecular weight forms (24, 23, 22 kDa), but not the 18kDa form of bFGF, are thought to initiate from novel CUG (leucine) codons, rather than



from the classical AUG (methionine) codon (Florkiewicz & Sommer 1989). It has been suggested that alternative translation initiation could serve as a postranscriptional mechanism for regulating bFGF gene expression and intracellular localization (Florkiewicz et al., 1991b). It is not known whether the three larger proteins have the same or different bioactivities as the 18 kDa protein. However, the data of Florkiewicz & Sommer (1989) show that the three higher molecular weight isoforms localize exclusively to the nucleus while the 18 kDa isoform is the only isoform exported onto the cell surface as well as colocalizing in the nucleus. Thus, it is possible that the different isoforms of human bFGF may have different functions depending upon their intracellular localization. In addition, truncated forms of bFGF, presumably the result of enzymatic degradation, have been purified from various tissues. For example, bFGF has been characterized as a 131 amino acid protein from ovary, kidney and adrenal tissue and as a 146 amino acid protein from brain and pituitary extracts (Burgess & Maciag 1989; Baird & Bohlen 1990).

Hitherto, the only postranslational modification to be described for aFGF and bFGF is the acetylation of the amino terminal alanine of full length human and bovine aFGF (1-154) and bFGF (1-154). There is no evidence to suggest that glycosylation of the potential N-linked glycosylation sites in human aFGF and bFGF actually occurs.

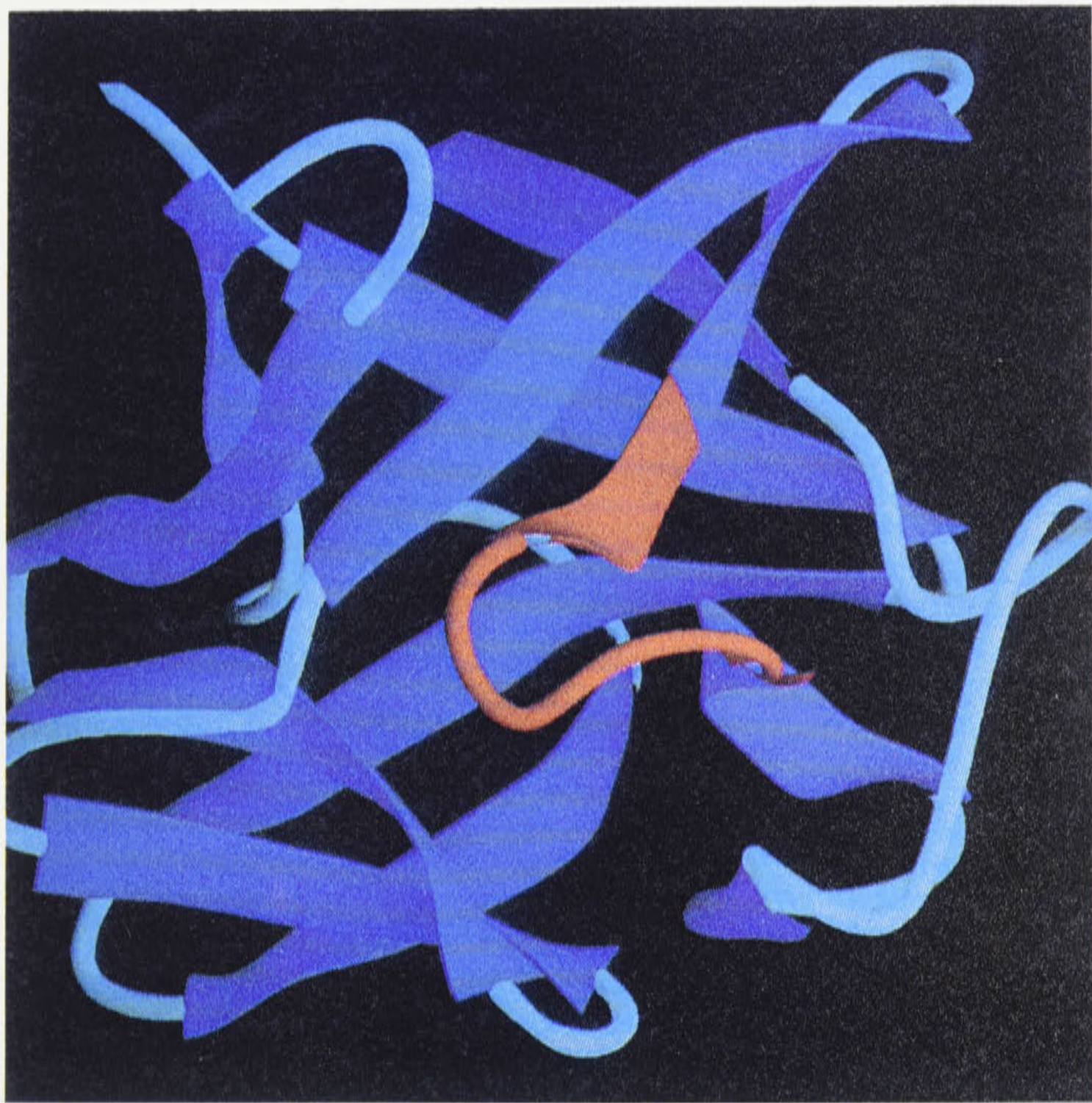
#### **1.5.2. Binding Sites on FGFs**

FGFs bind to FGF receptors (FGFR) on cell surfaces and Baird et al., (1988) have localized the receptor binding domain on FGF to a peptide spanning residues 106-115 of bFGF (Fig.1.1). This fragment begins in the middle of the 9th  $\beta$  strand, forms an open loop on the surface of the bFGF molecule and terminates at the beginning of the 10th  $\beta$  strand. One would expect the receptor-binding domain to be conserved between aFGF and bFGF since they bind to similar receptors. However, aFGF has two additional amino acids



FIGURE 1.1: Ribbon diagram illustrating the tertiary and secondary structure of bFGF.  $\beta$ -Sheet secondary structure is depicted with flattened ribbons. The polypeptide segment (residues 105-115) implicated in receptor recognition is shown in red. The diagram was produced using the computer program RIBBONS by M. Carson, University of Alabama, Birmingham. (From Zhang et al. 1991).







inserted at or near the position corresponding to the 102-113 peptide bond of bFGF with the result that the loop in aFGF corresponding to the 106-115 fragment of bFGF does not have the same backbone conformation. Furthermore, modulation of receptor affinity has been observed to occur as a result of phosphorylation of bFGF at Thr-112 by protein kinase A. Thr-112 lies within this putative receptor-binding domain and upon its phosphorylation, which is blocked in the presence of heparin, the affinity of bFGF for its receptor is increased by a factor of 3-8 fold (Feige & Baird 1989). This modulation in affinity may be the result of electrostatic interactions with Thr(P)-112 or due to conformational changes induced by phosphorylation (Zhang et al.,1991). Two nearby arginine residues, -107 and -109, have been proposed to act as potential ligands for the phosphothreonine (Zhang et al.,1991).

FGFs also bind to heparin and x-ray crystallographic analysis indicates that the presumed FGF receptor-binding region is about 25 Angstroms from the presumed heparin binding domain and that the two sites are on different faces of the molecule (Eriksson et al.,1991). This suggests that FGF binding to its receptor and FGF binding to heparin are likely to be independent events, supported by the observation that neutralizing antibodies inhibit the binding of bFGF to its receptor but not to heparin (Kurokawa et al.,1989).

While bound to heparin, aFGF and bFGF are protected from inactivation by high temperatures, acidic conditions and proteolysis (Gospodarowicz & Cheng 1986; Rosengart et al.,1988; Saksela et al.,1988; Mueller et al.,1989; Sommer & Rifkin 1989). Without native heparin, aFGF is relatively unstable at physiological temperatures at 35-45°C undergoing large structural changes and loss of mitogenic activity. However, a three-fold weight excess of heparin can increase the melting temperature by 20°C (Copeland et al.,1991). It has been suggested that heparin stabilizes aFGF in its native conformation (Gospodarowicz & Cheng 1986) rather than altering its conformation. Upon



binding to heparin, the thermal stability of bFGF is enhanced, raising the melting or transition temperature from 57°C to 82°C (Prestrelski et al.,1992). In contrast to aFGF, the binding of heparin to bFGF causes a conformational change in the molecule (Prestrelski et al.,1992). Heparan sulfate (HS), dextran sulfate and sucrose octasulfate also bind bFGF and induce a conformational change similar to that observed with heparin (Prestrelski, et al.,1992). The binding of heparin is thought to decrease the conformational flexibility of the bFGF molecule, or the steric hindrance from the bound heparin may lower the accessibility of the backbone amide hydrogens (Prestrelski et al.,1992). The interaction between FGF and heparin/HSPGs and its importance in the interaction of FGF with FGF receptors will be considered in more detail later (section 1.7.4.4).

Basic FGF, but not aFGF (Thomas & Gimenez-Gallego 1986), contains two Asp-Gly-Arg or DGR sequences which are the inverse of the cell adhesion sequence RGD (Presta et al.,1991). Generally peptides containing the RGD or DGR sequence can inhibit the interaction of integrins with ligands (Akiyama & Yamada 1985; Humphries et al.,1986; Hynes 1987; Ruoslahti & Pierschbacher 1987) such as FN, laminin, vitronectin and collagen (Ruoslahti & Pierschbacher 1986). It is hypothesized that bFGF may interact via its DGR sequences with an integrin-like molecule to affect bFGF-induced mitogenesis (Presta et al.,1991).

Both aFGF and bFGF interact with copper, enabling their purification by copper bioaffinity chromatography (Shing 1988; Yuen et al.,1990). Recombinant human forms of the aFGF monomer protein completely convert to aFGF homodimers after exposure to copper (Engleka & Maciag 1992). Such homodimers fail to induce mitogenesis, an effect reversed by dithiothreitol (Engleka & Maciag 1992), and have a reduced affinity for immobilized heparin, eluting with 0.4M NaCl. Interestingly, human bFGF's mitogenic activity and



affinity for heparin are relatively unaffected after treatment with copper. It is suggested that this difference between aFGF and bFGF may be due to differences in free thiol content and arrangement (Engleka & Maciag 1992).

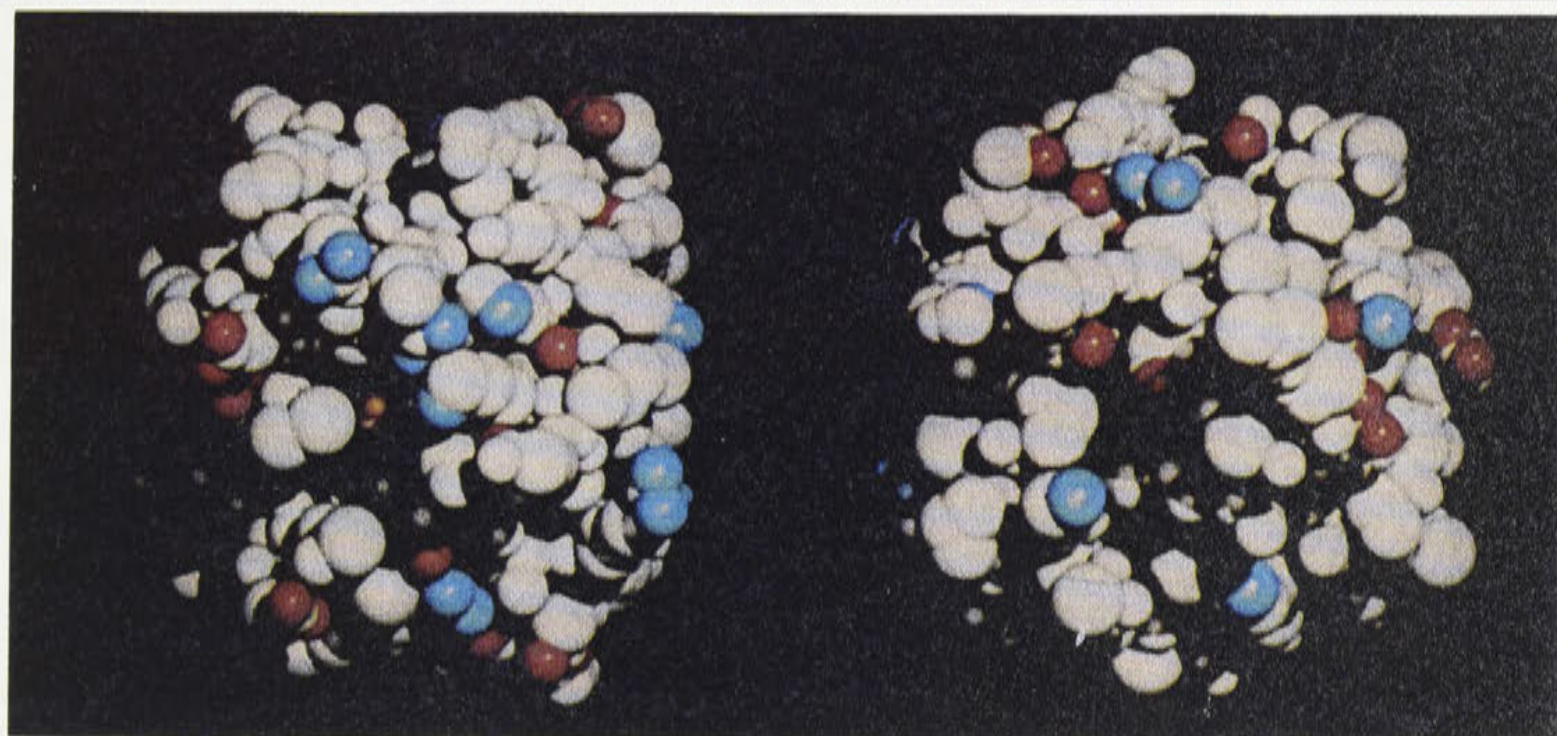
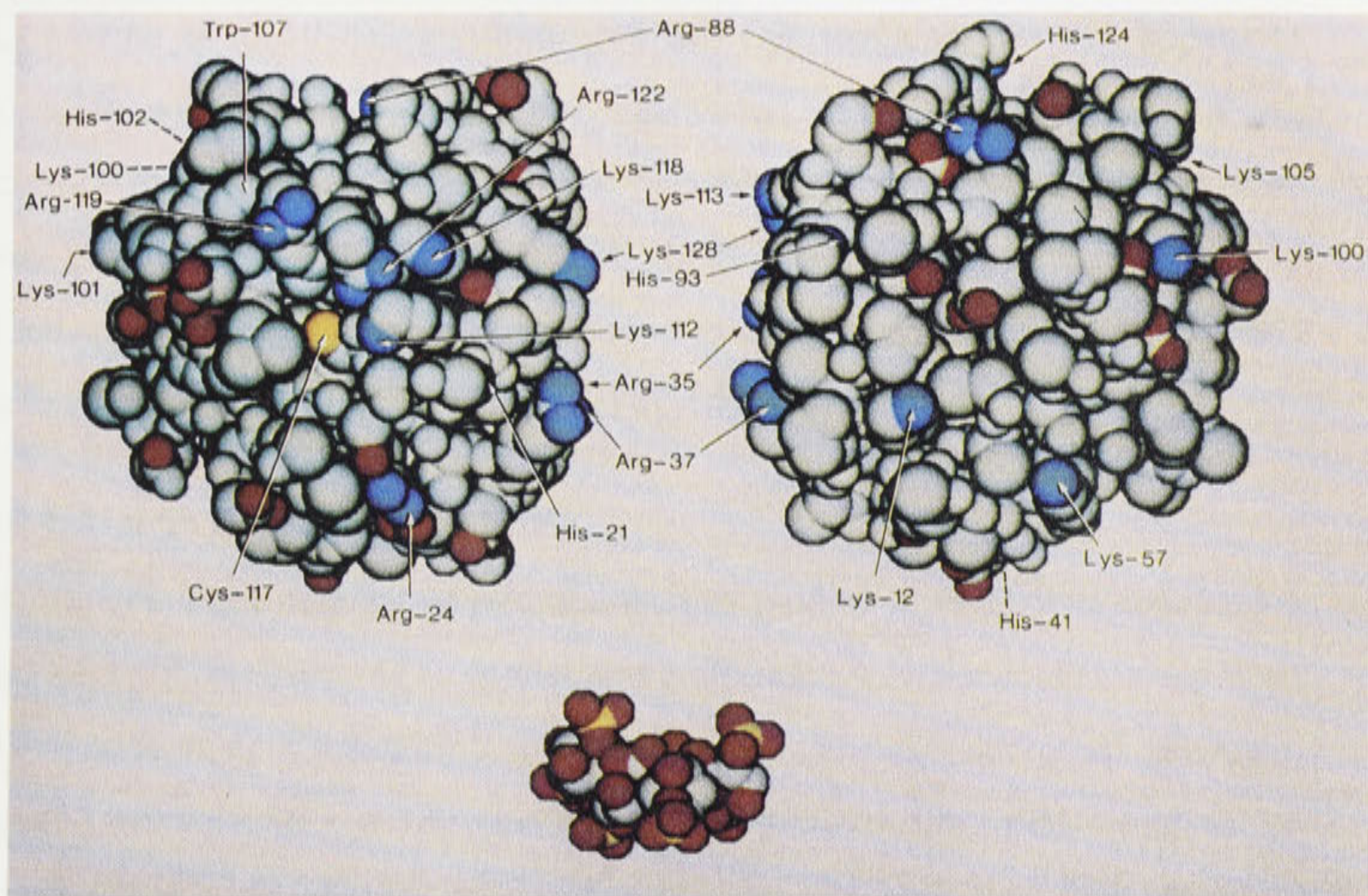
### 1.5.3. Three Dimensional Structure of FGFs

The three dimensional structures of bFGF (Fig. 1.1) and aFGF (Fig. 1.2) are similar, both structures consisting of 12 antiparallel  $\beta$  strands arranged in a pattern with approximately 3-fold internal symmetry (Zhu et al.,1990).  $\beta$  strands 1,4,5,8,9, and 12 (numbered sequentially from amino terminus) form a six-stranded antiparallel  $\beta$  barrel, closed at one end by  $\beta$  sheet interactions. There are extended loops between strands  $\beta$ 3 and  $\beta$ 4, between  $\beta$ 7 and  $\beta$ 8, and between  $\beta$ 11 and  $\beta$ 12. The main difference in the structures of aFGF and bFGF lies in the ordering of the nine residues at the amino terminus of aFGF and the deletion of two residues in bFGF corresponding to residues 105 and 106 in a turn between two  $\beta$  strands (Zhu et al.,1990). The amino terminal region of aFGF has been proposed as a nuclear translocation sequence (Imamura et al.,1990) and the crystal structures of FGF are consistent with this proposal. X-ray crystallography has enabled bFGFs overall structure to be described as a trigonal pyramid with a fold very similar to that reported for IL- $\alpha$ , IL-1 $\beta$  and soybean trypsin inhibitor (Eriksson et al.,1991). The core of the bFGF molecule is packed with hydrophobic and aromatic amino acid side chains, while the surface of the molecule is rich in the charged amino acids arginine and lysine (Zhang et al.,1991). Basic FGF has four cysteine residues, located at positions 25, 69, 87 and 92 (Eriksson et al.,1991). Cysteines -25 and -92 are conserved throughout the FGF family (Burgess & Maciag 1989) and are not involved in disulfide bridges. Examination of the crystal structure of bFGF shows that the cysteine residues are not located near enough to form a disulfide bond (Eriksson et al.,1991). However, bFGF does form oligomers, particularly dimers, under nonreducing conditions and thus dimerization may occur between Cys-69 and Cys-87 (Fox et al.,1988).



FIGURE 1.2: Space-filling models of aFGF and a typical heparin tetrasaccharide. In the upper panel, the two views are 180°  $\gamma$ -rotations of aFGF. The charged side-chain nitrogen atoms of basic residues are coloured blue; the side-chain acidic oxygen atoms are coloured red, and the cysteine sulfur atom is coloured yellow. All basic residues are labeled, along with the exposed Cys117 and Trp107. A representative heparin tetrasaccharide is shown below. The lower panel is a ray-traced rendering of the above view that illustrates the presence of the striking cavities on the surface of aFGF (From Volkin et al 1993).







Similarly in aFGF, disulfide bonds are not formed between the two cysteines at positions 16 and 83 (Linemeyer et al.,1990). In fact the forced formation of a disulfide bond between Cys-16 and Cys-83 in aFGF by copper oxidation resulted in a complete loss of activity which could be restored upon reduction (Linemeyer et al.,1990). However, the two Cys residues in aFGF appear to correlate with selective and rapid inactivation of mitogenic activity in the absence of heparin but afford a longer half-life in its presence. Therefore, they may play a role in the regulation of aFGF activity by promoting the rapid inactivation of aFGF when it is not bound to HSPGs on selected cell surfaces as in basement membranes (Ortega et al.,1991).

#### **1.5.4. Tissue Distribution of FGFs**

Differences in the qualitative and quantitative profiles of FGF gene expression and the tight regulation of its spatial and temporal synthesis would be expected to influence its activities during normal cell growth and development. However, despite the ubiquitous distribution of aFGF and bFGF, the mRNA required for their synthesis is not easily detected in most tissues and alternative means of regulation have been proposed. For example, it is suggested that their availability is determined by the target cell altering its expression of high affinity FGF receptors, rather than by gene expression (Baird & Walicke 1989) and that FGFs may be held in a storage form, bound to HSPG, in the ECM or on the cell surface until required. Furthermore, damage or degradation of the ECM could decrease FGFs affinity for the ECM resulting in increased availability for FGF receptors on neighbouring cells. However, Nurcombe et al.,(1993) provide evidence that FGF activity during mouse neural precursor development is regulated in part, by differential gene expression. Basic FGF mRNA was easily detected at embryonic day 9 (E9) and throughout subsequent ages whereas aFGF mRNA was only detectable at E11 and later.



### 1.5.5. Secretion of FGF

Unlike the other members of the FGF family, aFGF and bFGF lack a signal sequence normally required for transit through the secretory pathway. This has raised the question as to if, and how, aFGF and bFGF are released from living cells. Three models have been proposed to explain how FGF is released from the cell.

Firstly, FGF may be released after injury to the cell. Clearly, FGF can be released by lethal cell injury, induced for example, by endotoxin and by irradiation. Interestingly, irradiation produced an increase in *de novo* synthesis of the growth factor, peaking 72 hours following radiation treatment (Witte et al., 1989). An interesting question is whether transient sublethal cell injury is sufficient to release significant amounts of FGF. McNeil and Ito (1989) proposed that membrane disruption might be a common occurrence *in vivo* and that plasma membrane wounding followed by resealing might enable molecules to move in and out of the cytoplasm. Endothelial cell plasma membranes were transiently injured by scraping and the conditioned media assayed for FGF-like activity (McNeil et al., 1989). They suggest that transient cell injury is sufficient to release FGF but whether or not it occurs *in vivo* has yet to be demonstrated (McNeil, et al., 1989).

There is some evidence though, to suggest that bFGF is released from living cells which enables it to act as an autocrine factor. Rifkin, et al., (1991) utilized the phagokinetic assay originally developed by Albrecht-Buehler (Albrecht-Buehler 1977) to show that bFGF is in fact released from living cells and can act as a true autocrine factor (Quarto et al., 1991). Studies have shown that large vessel, small vessel and corneal endothelial cells all synthesize bFGF (Schweigerer et al., 1987c; Vlodavsky et al., 1987a,b). In the case of corneal endothelial cells, a majority of the FGF remains cell associated or in the ECM



(Vlodavsky et al., 1987a,b). Similarly for capillary endothelial cells, nearly all of the bFGF is cell associated (Schweigerer et al., 1987c). However, a measurable amount of bFGF in the conditioned media of adrenal cortex derived capillary endothelial cells has been shown to influence endothelial cell growth (Schweigerer et al., 1987a).

Addition of a signal peptide to bFGF and transfection of cells with this construct, results in cells with a transformed morphology along with tumorigenic and metastatic potential. It is therefore proposed that bFGF's lack of a signal peptide prevents it from being involved in autocrine stimulatory loops leading to transformation (Yayon & Klagsbrun 1990).

A second and alternative mode of FGF release could be similar to that described for a lectin from C2 mouse muscle cells which also lacks a signal peptide sequence and is also detected in the ECM (Cooper & Barondes 1990). This lectin is concentrated in patches beneath the plasma membrane which evaginate, producing extracellular vesicles containing the lectin. The extracellular vesicles release the lectin and it is proposed that the released material would then be able to bind to the cell surface and/or ECM. Such a release mechanism could help prevent excessive autocrine stimulation by FGF.

The third model is based on the observation that migrating cells are known to leave behind "bits" of cytoplasm. The continuous deposition of FGF-containing cytoplasm could lead to an accumulation of extracellular FGF (D'Amore 1990a).

### **1.6. FGF Receptors**

The mitogenic response initiated by FGFs is mediated by a plasma-membrane bound high affinity receptor. Normal diploid cells which are FGF-sensitive have a low density of FGF receptors, ranging from  $10^3$ - $10^4$  receptors/cell (Neufeld &



Gospodarowicz 1985). In contrast, established cell lines have a much higher density of FGF cell surface receptors (Neufeld & Gospodarowicz 1985,1986). For example, on BHK-21 cells aFGF binds its receptor with a  $K_d$  of 25 pM and there are 87,000 binding sites/cell (Neufeld & Gospodarowicz 1986) whereas for the same cell type bFGF has a  $K_d$  of 27 pM and 120,000 binding sites/cell (Neufeld & Gospodarowicz 1985). Binding of the FGFs to their receptors induces receptor down regulation and internalization of the ligand (Schreiber et al.,1985; Friesel et al.,1986; Huang et al.,1986; Clegg et al.,1987). Surprisingly, once internalized aFGF is relatively resistant to degradation (Kan et al.,1988; Friesel & Maciag 1988), the degradation products not appearing until two-three hours after internalization (Friesel & Maciag, 1988).

Cells also express a large number of low affinity binding sites for FGFs, determined to be HSPGs located on the cell surface or in the ECM (Moscatelli 1987,1988; Bashkin et al.,1989). FGFs bind to these low affinity sites with a  $K_d$  of between 2 and 10nM and can be removed by incubation with heparin, washing with 2M NaCl or by treatment with heparinase (Moscatelli 1987). The importance of these low affinity sites in the binding of FGFs to their high affinity receptor will be discussed later on (see section 1.7.4).

Initial studies using iodinated FGFs demonstrated that they bound with high affinity to receptors with  $K_d$ s of 50-500pM for aFGF (Schreiber et al.,1985; Baird et al.,1986; Libermann et al.,1987) and 10-200pM for bFGF (Neufeld & Gospodarowicz 1985; Moenner et al.,1986; Olwin & Hauschka 1986; Moscatelli 1987). The hypothesis that different members of the FGF family shared a common receptor was supported when it was shown that hst/KFGF caused a down regulation of high affinity binding sites for bFGF, indicating that bFGF and hst/KFGF can cross react with the same receptor (Moscatelli & Quarto 1989). Crosslinking experiments with aFGF and bFGF led to the detection of receptor species in the molecular weight range of 125-165 kDa (Neufeld &



Gospodarowicz 1985, 1986; Friesel et al., 1986; Moenner et al., 1986; Olwin & Hauschka 1986, 1989; Liebermann et al., 1987; Courty et al., 1988) with the two most prominent bands seen being 125kDa and 145kDa proteins. Acidic and basic FGF were able to bind to both the 125kDa and 145kDa proteins, bFGF having a higher affinity than aFGF for the 145kDa receptor species whilst aFGF had a higher affinity than bFGF for the 125kDa species (Neufeld & Gospodarowicz 1986; Olwin & Hauschka 1986). Although it was initially thought that the 125kDa protein represented a proteolytic cleavage product of the 145 kDa protein, it is now known that the two proteins are derived from different alternately spliced forms of FGF receptor mRNA.

The FGF receptors belong to the protein-tyrosine kinase superfamily of proteins (Fig.1.3). The observation that they contain immunoglobulin-like binding domains, also present in *Drosophila*, suggests that Ig-like binding domains in cell receptors preceded the immune system in vertebrates. It appears that the novel mechanisms which have evolved to generate diversity and secreted isoforms, are the same mechanisms which were later improved upon to produce the great diversity amongst immunoglobulin genes (Givol & Yayon 1992).

#### **1.6.1. Structural Features of FGF Receptors**

The different FGFR genes and their splice variants have been described in the literature under many different names. Table 1.5 should help to clarify some of this confusing nomenclature.

The first complete FGF receptor cDNA, denoted FGFR1, was isolated in 1989 by Lee et al., (1989) and since then three more distinct FGF receptor genes have been identified, namely, FGFR2, FGFR3 and FGFR4 (Fig.1.4). In general, the structures of FGFR3 and FGFR4 are very similar to those of FGFR1 and FGFR2. Although alternative splicing of the genes for FGFR1 and



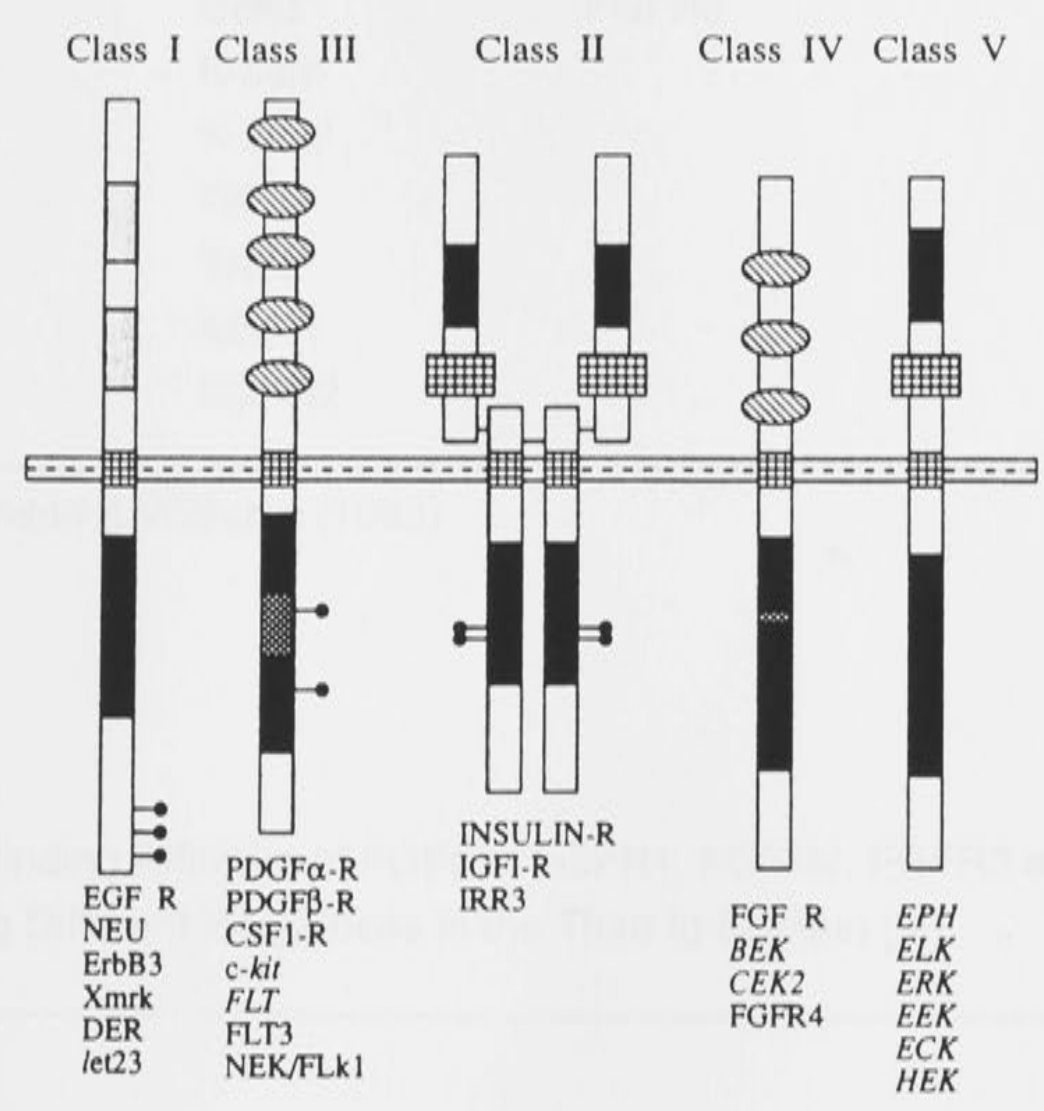
FIGURE 1.3: Receptor tyrosine kinase families. Five classes of receptor tyrosine kinase are shown, displaying structural features which define each class. The nature of each of these structural features is shown below. Classes I, II and III are as defined by Yarden & Ullrich (1988). (From Wilks 1993).



Table 1.3

Homodimeric and Heterodimeric Tyrosine Kinase Receptors

EGFR1	FGFR1	FGFR2	FGFR3	FGFR4
EGFR2				
EGFR3				
EGFR4				
EGFR5				
EGFR6				
EGFR7				
EGFR8				
EGFR9				
EGFR10				
EGFR11				
EGFR12				
EGFR13				
EGFR14				
EGFR15				
EGFR16				
EGFR17				
EGFR18				
EGFR19				
EGFR20				
EGFR21				
EGFR22				
EGFR23				
EGFR24				
EGFR25				
EGFR26				
EGFR27				
EGFR28				
EGFR29				
EGFR30				
EGFR31				
EGFR32				
EGFR33				
EGFR34				
EGFR35				
EGFR36				
EGFR37				
EGFR38				
EGFR39				
EGFR40				
EGFR41				
EGFR42				
EGFR43				
EGFR44				
EGFR45				
EGFR46				
EGFR47				
EGFR48				
EGFR49				
EGFR50				
EGFR51				
EGFR52				
EGFR53				
EGFR54				
EGFR55				
EGFR56				
EGFR57				
EGFR58				
EGFR59				
EGFR60				
EGFR61				
EGFR62				
EGFR63				
EGFR64				
EGFR65				
EGFR66				
EGFR67				
EGFR68				
EGFR69				
EGFR70				
EGFR71				
EGFR72				
EGFR73				
EGFR74				
EGFR75				
EGFR76				
EGFR77				
EGFR78				
EGFR79				
EGFR80				
EGFR81				
EGFR82				
EGFR83				
EGFR84				
EGFR85				
EGFR86				
EGFR87				
EGFR88				
EGFR89				
EGFR90				
EGFR91				
EGFR92				
EGFR93				
EGFR94				
EGFR95				
EGFR96				
EGFR97				
EGFR98				
EGFR99				
EGFR100				



- = Fibronectin type III repeat
- = Cysteine Rich Domain I
- = Cysteine Rich Domain II
- = Cysteine Rich Domain III
- = Ig-like Domain
- = Trans-membrane Domain
- = Tyrosine Kinase Domain
- = Insert Domain
- = Ligand-dependent Autophosphorylation Sites



Table 1.5:

Nomenclature of the Different FGF Receptor Genes

FGFR1	FGFR2	FGFR3	FGFR4
flg	bek	Cek2	FGFR4
bFGFR	Cek3	FGFR3	
Cek1	K-sam		
N-bFGFR	K-sam'		
h2, h3	TK14		
h4, h5	TK25		
FGFR1	KGFR		
	FGFR2		

From Johnson &amp; Williams (1993)

Table 1.6:

Relative Binding Affinities of FGFs to FGFR1, FGFR2, FGFR3 and FGFR4 Containing Different Sequences in the Third Ig Domain (III)

FGFR1	
IIIa	bFGF>aFGF
IIIb	aFGF>bFGF
IIIc	aFGF=bFGF
FGFR2	
IIIb	aFGF=KGF>bFGF
IIIc	aFGF=bFGF (KGF does not bind)
FGFR3	
IIIc	aFGF=bFGF
FGFR4	
IIIc	aFGF (bFGF does not bind)

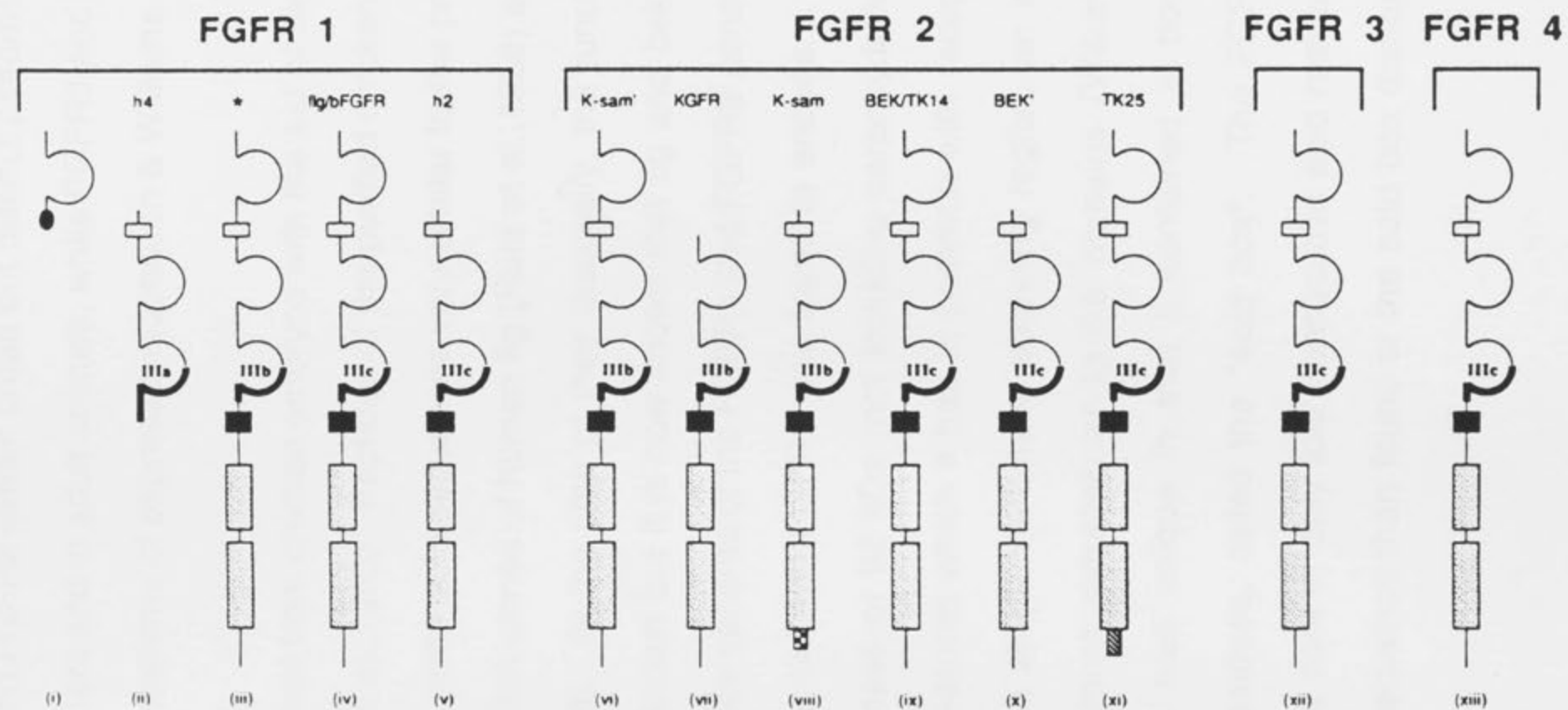
From Johnson &amp; Williams (1993); Keegan et al.,(1991); Partanen et al.,(1991).



FIGURE 1.4: Schematic diagram of FGF receptor protein structures. The figure shows the structure of variant receptor forms predicted by published cDNAs. The names of some receptor variants as they appear in the literature are written directly above the structure. Although cDNAs encoding the receptor variant depicted by an asterisk (iii) have not been isolated, PCR and Northern blotting experiments have identified mRNA transcripts encoding this receptor form (Johnson et al., 1991; Werner et al., 1992). Furthermore, both 3 Ig and 2 Ig domain forms of this receptor mRNA appear to exist. The following structural features are identified in the figure: the 32 unique amino acids at the C-terminus of the FGFR1 Ig domain 1 secreted form (solid oval), acid box domains (open boxes), alternative sequences for the second half of Ig domain III labeled IIIa, IIIb, or IIIc (thick black line), transmembrane domains (solid boxes), kinase I and kinase 2 domains (stippled boxes), and the unique C-tail domains of 2 FGFR2 proteins (checkered box and striped box).

(From Johnson & Williams 1993)





FGFR2, gives rise to multiple forms of the receptor, at this stage, only single cDNAs have been isolated for FGFR3 and FGFR4, both encoding 3 Ig domain receptor forms (Keegen et al.,1991; Partanen et al.,1991). Studies utilizing Northern blotting, RNase protection analyses and *in situ* hybridization have shown that FGFR1 and FGFR2 exhibit broad but distinct patterns of expression both during development and in adult animals, while FGFR3 and FGFR4 genes have more restricted patterns of expression (Johnson & Williams 1993).

FGFR1 was first purified from chicken embryos with the aid of biotinylated FGF and heparin (Kiefer et al.,1990). Peptides of the purified chicken FGF receptor were shown to have amino acid sequences very similar to the predicted amino acid sequences of cDNA clones of human flg (Ruta et al.,1988) and mouse bek (Kornbluth et al.,1988). At the time of their discovery, the function of flg and bek proteins was unknown but it is now known that flg and bek cDNA clones represent specific splice variants of the FGFR1 and FGFR2 genes respectively. A cDNA encoding the chicken FGFR1 was found to encode a protein with a deduced molecular mass of 92 kDa (not including carbohydrate side chains) (Lee et al.,1989). Features which it had in common with receptors for other growth factors were a single membrane spanning region, an amino-terminal signal peptide, and three extracellular Ig-like domains (Williams & Barclay 1988). However, it was unique in that it contained a domain of eight consecutive acidic residues, called the "acid box". The FGFR4 protein is unique in containing a core of only four consecutive acid residues in the acid box, a shorter core sequence than found in the acid box domains of FGFR1, FGFR2 and FGFR3.

The presence of consensus tyrosine kinase sequences confirmed the receptor as a tyrosine-specific protein kinase (Huang & Huang 1986; Coughlin et al.,1988). The tyrosine kinase sequence is split by an insertion of 14 amino acids and is considerably shorter than those of PDGF- $\beta$  (Yarden et al.,1988)



and CSF-1 (Coussens et al.,1986) receptors but similar to those of insulin and insulin-like growth factor-1 receptors (Ullrich et al.,1985, 1986) (Fig.1.5).

A most interesting discovery has been the isolation of multiple, distinct cDNAs encoding variant forms of FGFR1 and FGFR2 (Dionne et al.,1990; Johnson et al.,1990; Reid et al.,1990; Champion-Arnaud et al.,1991; Eismann et al.,1991; Hou et al.,1991; Miki et al.,1991). It now seems that alternative splicing of mRNA is responsible for the diverse receptor forms (Champion-Arnaud et al.,1991; Johnson et al.,1991), many more than seen for any other growth factor receptor. Alternative splicing may result in either a) the inclusion/exclusion of additional amino acids or b) the use of alternate coding exons with no net gain or loss of amino acids, in both cases producing structurally different proteins.

Since the first reported FGFR1 cDNA was shown to encode a protein with 3 Ig domains, cDNAs encoding FGFR1 proteins that are missing Ig domain 1 have been found (Johnson et al.,1990; Mansukhani et al.,1990; Reid et al.,1990) apparently produced by alternative splicing (Fig.1.4). The function of the Ig domain 1 is currently unknown but it does not appear to be necessary for high affinity binding of aFGF and bFGF as the 3 Ig domain form of FGFR1 has been shown to have binding affinities for aFGF and bFGF (Dionne et al.,1990; Johnson et al.,1990) similar to those of the 2 Ig domain form of FGFR1 (Johnson et al.,1990). cDNAs encoding 3 Ig domain and 2 Ig domain forms of FGFR2 have also been identified (Dionne et al.,1990; Hattori et al.,1990; Houssaint et al.,1990; Champion-Arnaud et al.,1991; Crumley et al.,1991; Miki et al.,1991) and both forms exhibit similar high affinities for aFGF and bFGF (Dionne et al.,1990; Crumley et al.,1991). Thus, as is the case with FGFR1, Ig domain 1 of FGFR2 does not seem to be required for high affinity binding of aFGF and bFGF.

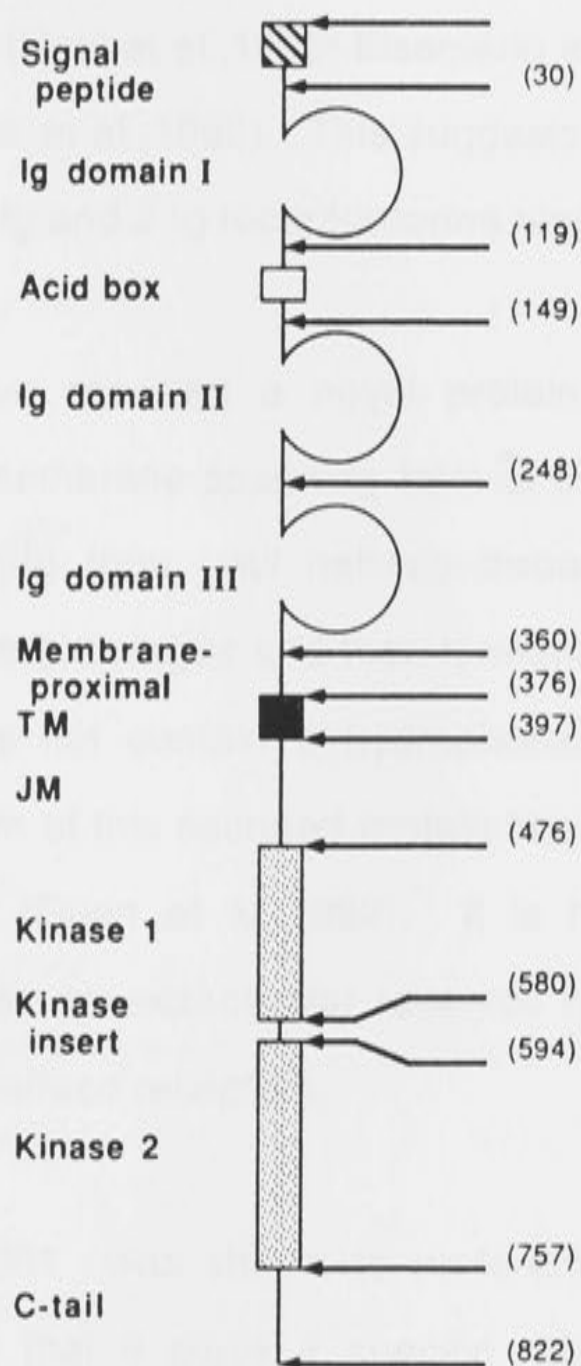


FIGURE 1.5: Structural features of the FGFR1 protein. The numbers in parentheses indicate the last amino acid of each domain (From Johnson & Williams 1993).



As was mentioned previously, the early crosslinking studies with aFGF and bFGF identified two prominent polypeptides of 125 and 145 kDa. Recent studies (Dionne et al., 1990; Johnson et al., 1990; Mansukhani et al., 1990) have revealed that cross-linked receptors containing 3 Ig domains are about 145 kDa in size while cross-linked receptors containing 2 Ig domains are approximately 125 kDa in size. Coexpression of 3 Ig and 2 Ig mRNA transcripts have been detected in a variety of cell lines using PCR (Johnson et al., 1990; Eisemann et al., 1991), Northern blotting (Reid et al., 1990; Eisemann et al., 1991) and RNase protection analyses (Werner et al., 1992). This suggests that the 145 and 125 kDa bands represent the 3 Ig and 2 Ig receptor forms respectively.

Johnson et al., (1990) have reported a novel protein which represents a secreted form of the 2 Ig membrane-spanning form of FGFR1. This secreted form is identical to the 2 Ig form until halfway through the Ig domain III (Johnson et al., 1990) where it diverges and then terminates in 79 amino acids downstream. It also does not contain a hydrophobic membrane-spanning domain. A 3 Ig domain form of this secreted protein has also been discovered and shown to bind bFGF (Duan et al., 1992). It is hypothesized that the secreted FGFR could act as an extracellular reservoir of FGF, regulating the availability of FGFs to cell surface receptors.

The secreted form of FGFR1 was shown to preferentially bind bFGF over aFGF which may indicate that it plays a specific role in regulation bFGF function. The mouse counterpart of the secreted human form lacks the COOH-terminal half of the predicted third Ig-like domain (Werner et al., 1992) and binds both basic and acidic FGF with low affinity, Kds being greater than 100nM (Werner et al., 1992). Therefore the COOH-terminal half of the 3rd Ig-like domain may be required for determining the affinity and specificity for FGF ligands. Other secreted growth factor receptors generated by alternative



splicing of mRNA are the secreted IL-4 receptor (Mosley et al.,1989) and the secreted IL-7 receptor (Goodwin et al.,1990).

There are three alternative exons for the second half of Ig domain III in human FGFR1 and these have been denoted IIIa, IIIb and IIIc (Johnson et al.,1991). The FGFR2 gene has exons corresponding to IIIb and IIIc but not to IIIa, whilst FGFR3 and FGFR4 have only exons IIIc in their Ig domain III (Keegan et al.,1991, Partanen et al.,1991) (Fig.1.4). Comparisons of the IIIb and IIIc sequences of FGFR1 and FGFR2 show a greater divergence between similar exons (IIIb and IIIc of the same gene, than between corresponding exons of different genes), suggesting that the existence of the IIIb and IIIc exons is what enabled the existence of a multigene family (Johnson & Williams 1993). Binding experiments have shown that alternative splicing in the third Ig domain is important for determining receptor binding specificities for those FGFs that have been compared namely, aFGF, bFGF and KGF. Table 1.6 shows the effect of alternate splicing in the third Ig domain of FGFR1, FGFR2, FGFR3 and FGFR4 on the relative binding affinity of aFGF, bFGF and KGF.

#### **1.6.2. FGF Receptor Sequence Homology**

There is a striking degree of similarity in the amino acid sequences of FGFR1 proteins from different species. The mouse, chicken, and *Xenopus* FGFR1 proteins are 98%, 91% and 78% identical to the human FGFR1 protein. The most highly conserved regions of the receptor molecule are the kinase 1 and kinase 2 domains which exhibit 92% and 95% identity respectively with human and *Xenopus* whereas the least conserved regions are the signal peptide region and the Ig domain 1, the membrane proximal domain, the transmembrane domain and the kinase insert domain. This is a higher level of identity than observed among different members of the FGF family (35 to 55%). However, the pattern of conservation is similar to that observed when comparing FGFR1 domains across species. Table 1.7 shows the overall



Table 1.7:

Sequence Homology Between the Different Human FGF Receptor Genes<sup>a</sup>

	FGFR2	FGFR3	FGFR4
FGFR1	72%	62%	55%
FGFR2		66%	57%
FGFR3			61%

<sup>a</sup>Sequence homology in the 3 Ig domain receptor forms containing IIIc type sequences.

From Johnson & Williams (1993)

Table 1.8:

Tissue Distribution of FGF Receptors in Human Foetal Tissue

Receptor	Distribution	Reference
FGFR1	growth plate of bones, skin, brain-neuronal populations	Partanen et al.,(1991) Peters et al.,(1992)
FGFR2	choroid plexus, skin, lung, kidney, temporal lobe, brain-glial cells	Partanen et al.,(1991) Peters et al.,(1992)
FGFR3	intestine, lung, kidney, bone growth plates	Partanen et al.,(1991)
FGFR4	adrenal, lung, kidney, liver	Partanen et al.,(1991)

degree of amino acid identity of the IIIc type sequences in the 3 Ig domain form of the different human FGFR genes.

### 1.6.3. Tissue Distribution of FGF Receptors

Multiple members of the FGF family can bind to the same receptor species. Both aFGF and bFGF have been shown to bind to 3 Ig and 2 Ig FGFR1 forms with high affinity ( $K_d$  for aFGF of 20-80pM and 50-150pM for bFGF) (Dionne et al., 1990; Johnson et al., 1990) and to activate receptor tyrosine kinase activity and receptor mediated signalling (Dionne et al., 1990; Johnson et al., 1990; Mansukhani et al., 1990). Hst/KFGF also binds to both receptor forms but with lesser affinity than acidic and basic FGFs (Dionne et al., 1990; Mansukhani et al., 1990). Similarly, multiple members of the FGF family are able to bind to, and activate FGFR2 and FGFR3 proteins. This leads us to the question of how cells and tissues can selectively respond to individual members of the FGF family. Distinct receptors may be able to generate different cytoplasmic signals by differential tissue expression of the receptors and limited mobility of the ligands. FGFR1, FGFR2, FGFR3 and FGFR4 expression, when analyzed at the RNA level are found to be independently regulated in human fetal (17-18 week) tissues (Partanen et al., 1991) (see Table 1.8). Differential expression of FGFR1 and FGFR2 in mouse embryos was demonstrated by *in situ* hybridization which showed FGFR1 to be associated with mesenchymal tissues and FGFR2 with epithelium (Peters et al., 1992a; Ueno et al., 1992). The same pattern of expression has also been observed in embryonic tissues such as skin, limb, gut and lung (Peters et al., 1992a). The expression of the three FGFRs, cek-1 (FGFR1), cek-2 (FGFR2) and cek-3 (FGFR3), has also been shown to change during chick embryo development indicating their temporal regulation (Patstone et al., 1993). Rather than the distribution of FGFs matching a particular receptor, different FGFRs appear to be associated with particular cell types and will interact with whichever growth factor is present in its vicinity (Patstone et al., 1993). The spatial and temporal expression pattern



of the FGF receptors, along with their differential binding specificity is proposed as the means by which the function of the FGF receptors is controlled (Ornitz & Leder 1992).

Differential expression of the IIIa, IIIb and IIIc exons by FGFR1 in mouse tissues has been demonstrated (Werner et al.,1992). The IIIc exon was expressed simultaneously with more than one exon, with IIIc expression levels being much higher than those of IIIa or IIIb exons. Johnson et al., (1990) also found this to be the case in several human cell lines. Werner et al.,(1992) found exons IIIa and IIIb to exhibit more restricted patterns of expression; IIIa being expressed in brain, skeletal muscle and skin, and IIIb predominantly in skin but at low levels in brain, kidney, muscle and placenta.

Removal of the first Ig domain by alternative splicing has been shown to occur in a tissue-specific fashion. Three Ig domain forms of FGFR1 are the predominant form of receptor expressed during mouse embryogenesis (Reid et al.,1990; Werner et al.,1992) while 2 Ig domain forms are not detected by Northern blotting and RNase protection assays until after birth. After birth, 3 Ig and 2 Ig forms are simultaneously expressed in tissues such as heart, lung, and muscle (Werner et al.,1992). Three Ig domain forms, however, remain the predominant form in brain and kidney.

Tissue specificity of expression of alternatively spliced transcripts has also been shown to occur. KGFR and FGFR2 are both encoded by the same gene but KGFR, detected by a probe specific for exon IIIb, is associated with epithelial cells of skin and internal organs or ducts while FGFR2 (exon IIIc) is confined to bones and nonmesenchymal layers (P.Lonai-personal communication to Givol & Yayon 1992). These data are suggestive of a mechanism specific to cell-type, utilizing mutually exclusive alternative splicing. The different binding properties of keratinocytes and fibroblasts/endothelial



of the FGF receptors, along with their differential binding specificity is proposed as the means by which the function of the FGF receptors is controlled (Ornitz & Leder 1992).

Differential expression of the IIIa, IIIb and IIIc exons by FGFR1 in mouse tissues has been demonstrated (Werner et al.,1992). The IIIc exon was expressed simultaneously with more than one exon, with IIIc expression levels being much higher than those of IIIa or IIIb exons. Johnson et al., (1990) also found this to be the case in several human cell lines. Werner et al.,(1992) found exons IIIa and IIIb to exhibit more restricted patterns of expression; IIIa being expressed in brain, skeletal muscle and skin, and IIIb predominantly in skin but at low levels in brain, kidney, muscle and placenta.

Removal of the first Ig domain by alternative splicing has been shown to occur in a tissue-specific fashion. Three Ig domain forms of FGFR1 are the predominant form of receptor expressed during mouse embryogenesis (Reid et al.,1990; Werner et al.,1992) while 2 Ig domain forms are not detected by Northern blotting and RNase protection assays until after birth. After birth, 3 Ig and 2 Ig forms are simultaneously expressed in tissues such as heart, lung, and muscle (Werner et al.,1992). Three Ig domain forms, however, remain the predominant form in brain and kidney.

Tissue specificity of expression of alternatively spliced transcripts has also been shown to occur. KGFR and FGFR2 are both encoded by the same gene but KGFR, detected by a probe specific for exon IIIb, is associated with epithelial cells of skin and internal organs or ducts while FGFR2 (exon IIIc) is confined to bones and nonmesenchymal layers (P.Lonai-personal communication to Givol & Yayon 1992). These data are suggestive of a mechanism specific to cell-type, utilizing mutually exclusive alternative splicing. The different binding properties of keratinocytes and fibroblasts/endothelial



cells for FGF may well reflect differential expression of IIIb and IIIc exons in these cells. Basic FGF and not aFGF stimulates mitogenesis in human melanocytes (Halaban et al., 1987), and while aFGF and bFGF stimulate the proliferation of fibroblast and endothelial cells, KGF does not (Rubin et al., 1989). In contrast, KGF stimulates keratinocyte proliferation.

Differences in the affinity of the various FGFs for particular receptors is attributed to the differences between the genes, (possibly sequence differences between homologous domains), rather than to differential splicing leading to heterologous domain structures (Ornitz & Leder 1992). Keegan et al., (1991) suggest that each of the FGFRs is likely to have a specific ligand but may be able to bind more than one ligand at lower affinity and that each isoform of a particular receptor may also have different binding capacities for the same ligand (Table 1.6).

#### **1.6.4. FGFRs in Invertebrates**

Shishido et al., (1993) have identified two homologues of mammalian FGFR genes in the *Drosophila* genome and denoted them DFR1 and DFR2. DFR2 seemingly corresponds to DFGFR identified by Glazer & Shilo (1991), because it is expressed in endodermal precursors and is virtually identical in nucleotide sequence. Initially during embryogenesis, DFR1 is expressed in mesodermal primordium and DFR2 in the primordia of the anterior and posterior midguts. Later, DFR1 RNA is expressed in developing tissues derived from mesoderm and neuroectoderm while DFR2 RNA is expressed in ectoderm and mesoectoderm derived tissues. It is suggested that DFR1 and DFR2 genes may be derivatives of a common ancestral arthropod gene which diverged from an ancient vertebrate FGF-R gene when the major phylogenetic branch to the arthropods and chordates occurred (Glazer & Shilo 1991). DFR1 has been shown to have 2 Ig-domains and DFR2 to have 5 Ig domains and they may recognize different ligands.



### **1.7. Heparin/HSPG Interaction with FGF**

An important feature of acidic and basic FGF is their ability to bind to heparin and HS. Recent studies have demonstrated that this interaction is essential for the full biological activity of FGF to be expressed (Yayon et al., 1991; Rapraeger et al., 1991; Olwin & Rapraeger 1992; Mansukhani et al., 1992). This section will consider the heparin/HS -FGF interaction in considerable detail, particularly as this interaction is the subject of much of the research work presented in this thesis.

#### **1.7.1. Structural Features of Heparin and Heparan Sulfate**

Before discussing the interaction of heparin and HS with acidic and basic FGFs, I will briefly describe and compare the structural features of heparin and HS. Heparin and HS are GAGs and they share with the other GAGs namely, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid, a characteristic disaccharide repeat sequence, one monosaccharide of the disaccharide repeat being an amino sugar and the other, except in the case of keratan sulfate, a uronic acid residue (Jackson et al., 1991). With the exception of hyaluronic acid which is not sulfated, they are variably N- and O-sulfated (see Jackson et al., 1991 for review).

Heparin and HS are the most negatively charged structures produced by vertebrate cells. They are ubiquitously distributed throughout the animal phyla being present in chordates, mollusks, annelids and arthropods and also in animals as ancient as coelenterates (Nader et al., 1988). The most notable structural features of heparin and HS are their high negative charge density resulting from carboxyl and sulfate groups and their heterogeneous composition. Their unbranched carbohydrate backbone consists of either D-glucuronic (GlcA) or L-iduronic (IdoA) acid and D-glucosamine (GlcN) units joined by  $\alpha$ 1,4-glycosidic linkages (Kjellen & Lindahl 1991). Figure 1.6



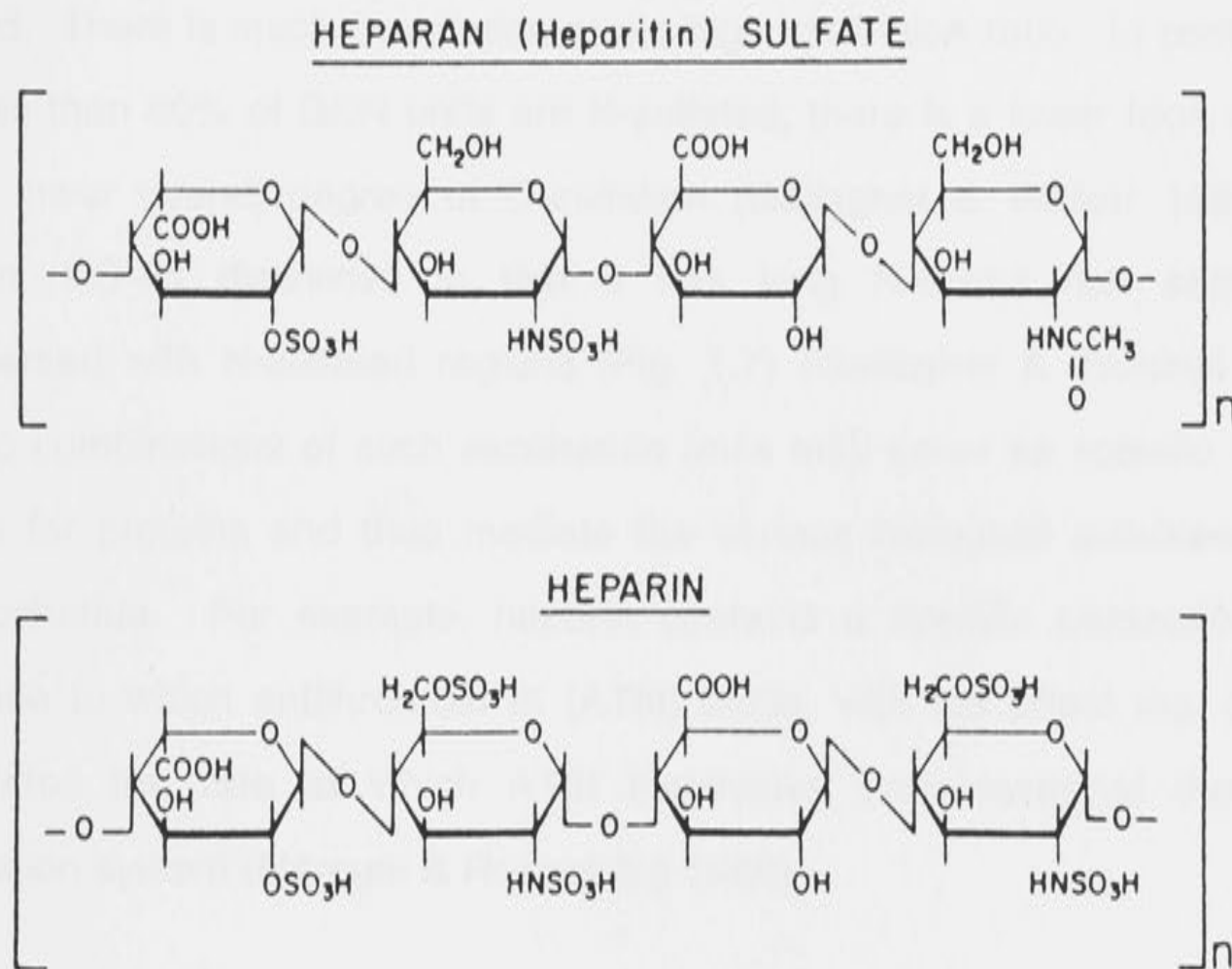


FIGURE 1.6: Typical oligosaccharide structures of heparin and heparan sulfate. (From Silbert 1982)

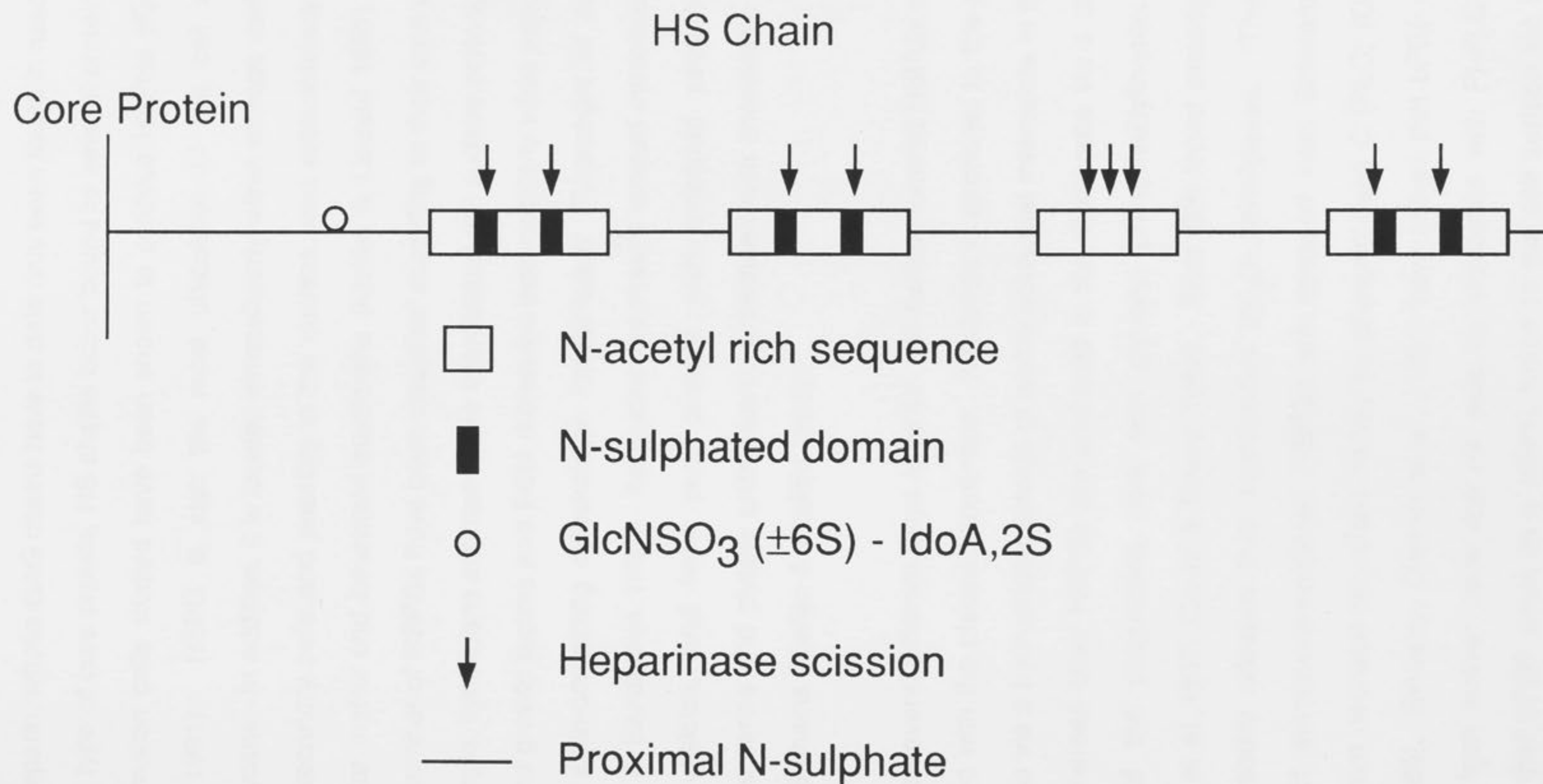


illustrates the typical disaccharide structures of heparin and HS. Due to the variable location of N-acetyl, N-sulfate and O-sulfate groups on these three constituent monosaccharides there are at least ten different monosaccharide building blocks which can be combined into a large number of different saccharide sequences (Lindahl 1989). Heparin and HS are composed of the same monomeric building blocks, the components being present in different proportions. In heparin, generally more than 80% of the GlcN residues are N-sulfated. There is much O-sulfation and a high IdoA/GlcA ratio. In contrast, in HS, less than 80% of GlcN units are N-sulfated, there is a lower IdoA content and a lower overall degree of O-sulfation (Gallagher & Walker 1985). In addition, HS is distinctive in that it has long N-acetyl rich sequences interspersed with N-sulfated regions (Fig. 1.7) (Gallagher & Turnbull 1992). Specific combinations of such saccharide units may serve as specific binding regions for proteins and thus mediate the various biological activities of the polysaccharide. For example, heparin contains a specific pentasaccharide sequence to which antithrombin III (ATIII) binds, with the effect that heparin accelerates the rate at which ATIII inactivates proteinases of the blood coagulation system (Marcum & Rosenberg 1989).

Heparin is synthesized by mast cells and stored within the cytoplasmic granules of these cells. In bovine liver capsule heparin occurs as single polysaccharide chains of molecular weight greater than 10,000 (Jansson et al., 1975), but in rat skin (Horner 1971) and in peritoneal mast cells (Yurt et al., 1977) it has a molecular weight of approximately  $1 \times 10^6$  and the GAG chains are attached to a core protein structure. Apparently, after heparin is released from mast cell granules it exists as free GAG chains, not associated with a core protein. Some tissues have been found to contain heparin in both forms (Ogren & Lindahl 1971) and it is thought that the single chain forms may be the result of cleavage of the polypeptide-bound chains by specific endoglycosidases at a limited number of sites (Ogren & Lindahl 1971; Horner 1977).

FIGURE 1.7: Domain structure of heparan sulfate. Heparan sulfate is believed to have a domain structure with N-sulfated regions interspersed with long N-acetyl-rich sequences. Heparanase cleavage sites are present in the sulfated domains. The most proximal (to the protein) heparinase site is ~16 disaccharides from the core protein, whereas the innermost N-sulfate is ~10 disaccharides from the protein. (From Gallagher & Turnbull 1992)





Unlike heparin, whose GAG chains have to date only been found in association with one type of core protein, HS chains occur bound to several proteins, and all mammalian cells studied have been shown to produce HSPGs (Kjellen & Lindahl 1991). HSPG is also the most ubiquitous of the cell surface proteoglycans. In addition, it is located intracellularly within storage vesicles of various secretory cells and possibly in the nucleus, and extracellularly in the pericellular matrix and basement membrane (Kjellen & Lindahl 1991) (Table 1.9). A number of HSPGs have been classified according to their core protein. For example, syndecans are membrane intercalated cell surface proteoglycans which bind growth factors and ECM molecules extracellularly while associating with the actin-containing cytoskeleton intracellularly (Rapraeger et al., 1987; Bernfield & Sanderson 1990). Serglycins are mainly located intracellularly in storage vesicles, and have core proteins with extended sequences of alternative serine and glycine units heavily substituted with chondroitin sulfate and/or HS chains (Kjellen & Lindahl 1991).

There are several different ways in which cell-surface-located HSPGs may be associated with the plasma membrane. Syndecan is anchored to the plasma membrane via a hydrophobic stretch of amino acids that intercalate in the lipid bilayer whereas other HSPGs are anchored in the membrane via a covalent linkage of the polypeptide core with glycosyl phosphatidylinositol (GPI) (Ishihara et al., 1987; Carey & Evans 1989). Such GPI-linked proteoglycans can be readily released from membranes by phospholipases. Thus, fifty percent of membrane-anchored HSPGs are released from Schwann cells derived from neonatal rat sciatic nerves by phospholipase C (PLC) (Carey & Evans 1989). Similarly, Brunner et al., (1991) have shown that bFGF can be released from human bone marrow cells by incubation with PI-PLC which indicates that bFGF binds to a HSPG linked to the cell surface via a GPI-anchor. A GPI-linked HSPG might provide a highly selective and efficient



Table 1.9: Heparan Sulphate and Heparin Proteoglycans

Proteoglycan type	MW	Glycosaminoglycans		Core protein MW	Cell/tissue expression
		Chain number	MW		
Cell surface HSPG	$7.5 \times 10^4$	4	$1.4 \times 10^4$	$3 \times 10^4$	Liver cell surface
	$3.5 \times 10^5$	4-6 per polypeptide	$2 \times 10^4$	$9 \times 10^4$ (dimer)	Fibroblast cell surface
	$1-2 \times 10^5$	ND	ND	$1 \times 10^5$	Synaptic vesicles
Basement membrane HSPG	$1.3-2.5 \times 10^5$	4-5	$2.5 \times 10^4$	$0.9-1.7 \times 10^5$	Glomerulus
	$>7.5 \times 10^5$	6-12	$7 \times 10^4$	$3.5 \times 10^5$	Engelbreth-Holm-Swarm sarcoma
	$4 \times 10^5$	10	$2.5 \times 10^4$	$1.5 \times 10^5$	PYS-2 teratocarcinoma cells
Heparin	$1 \times 10^6$	9-12	$0.5-1 \times 10^5$	$2.0 \times 10^4$	Mast cell

From Poole (1986)

mechanism for releasing active growth factor without otherwise perturbing the microenvironment (Brunner et al., 1991).

Alternatively, HSPGs may associate with the cell membrane via cell-surface receptors that specifically recognize core proteins or are held at the cell surface through noncovalent binding of the glycan side chains to a membrane receptor (Hook et al., 1984). Such receptors could participate in the endocytosis of extracellular proteoglycans. These heparan sulfates can be released from the membrane by inositol hexaphosphate, heparin or high salt concentrations which are thought to compete for the electrostatic interactions between the negatively charged HS side chains and the positively charged proteins at the cell surface (Carey & Evans 1989). Similarly, sulfated GAG molecules which are free of their core proteins have been identified as peripheral components of 3T3 fibroblast plasma membranes. Piepkorn et al., (1989) suggested that membrane-associated endoglycosidases may be responsible for the release of these peripheral GAGs from their core protein. In fact, Gallagher et al., (1988) have isolated and characterized a liver plasma membrane-associated HS endoglycosidase which may perform this function.

#### **1.7.2. Heparin Binding Domain of Acidic and Basic FGF**

In an attempt to further understand the role of heparin/HS in FGF action and the specificity of this interaction, as well as the interaction between FGF and its receptor, structure-function studies have concentrated on locating and defining the heparin-binding and receptor-binding domains of acidic and basic FGF.

Studies utilizing chemical modification, site-directed mutagenesis and synthetic peptides have implicated basic residues, in particular Lysine-118, in the heparin binding site of aFGF and bFGF (Baird et al., 1988; Harper & Lobb 1988; Lobb 1988a; Seno et al., 1990; Burgess et al., 1991) (See Table 1.10) Lysine residues have also been implicated in the interaction between heparin and



Table 1.10:  
Regions on FGFs Proposed as Heparin-Binding Domains

Analysis by:	Residues implicated	Reference
<u>Acidic FGF</u>		
reductive methylation	Lys-118	Harper & Lobb (1988)
site-directed mutagenesis	Lys-132	Burgess et al.,(1991)
synthetic peptides	49-71	Mehlman & Burgess (1990)
X-ray crystallography	105-128	Zhu et al.,(1991)
X-ray crystallography	Asn-18, Lys-113, Lys-118	Eriksson et al.,(1991)
X-ray crystallography	Lys-112, Lys-118, Arg-119, Arg-122	Volkin et al.,(1993)
<u>Basic FGF</u>		
Deletion mutagenesis	Thr <sup>105</sup> -Pro <sup>141</sup>	Seno et al.,(1990)
Synthetic peptides	24-68, 73-87, 93-120	Baird et al.,(1988)
X-ray crystallography	Lys-119, Arg-120, Lys-125, Lys-129	Zhang et al.,(1991)
X-ray crystallography	105-128	Zhu et al.,(1991)
X-ray-crystallography	Asn-27, Arg-120, Lys-125	Eriksson et al.,(1991)

platelet factor 4 (PF4; Deuel et al.,1977), ATIII (Rosenberg & Damus 1973), heparin cofactor II (Church & Griffith 1984) and between heparin and apolipoprotein E (Weisgraber et al.,1986). In fact, it has been proposed for many years that clusters of basic residues are important in protein-heparin interactions (Deuel et al.,1977). In the case of aFGF, there are two segments containing clusters of basic residues; lysines -9,-10, and -12 near the amino terminus and the region spanning residues 100 to 122. Chou and Fasman (1978) used secondary structure analysis of aFGF to demonstrate that Lys-118 is contained within a  $\beta$ -turn region encompassing residues 112-123 which probably form part of the surface of the protein (Fig. 1.2). A similar loop structure is also predicted for human aFGF and for human and bovine bFGF.

X-ray crystallographic studies of aFGF and bFGF implicate Lys-112, Lys-118 and Arg-122 (in human aFGF) in the heparin binding site of both FGFs (Zhu et al.,1991; Eriksson et al.,1991; Zhang et al.,1991). Volkin et al.,(1993) utilized the ability of various polyanions to stabilize the conformation of aFGF, in order to investigate the nature of its polyanion binding site. Their three dimensional model of human aFGF shows Lys-112, Lys-118, and Arg-122 form a tight cluster of positively charged sidechains. Close by, and partially solvent exposed is Cys-117 and it is likely that polyanions protect the thiol groups from metal catalyzed oxidation. The deep cavity in which Cys-117 sits probably comprises part of the polyanion binding site and polyanions may stabilize aFGF through occlusion of the clefts within this cavity (Fig.1.2). The authors conclude that the major polyanion binding site of aFGF probably extends from Arg-119 across the surface of the molecule to the three clustered positively charged residues. In this context, the ability of high concentrations of neutral salts to completely inhibit polyanion binding suggests a binding site of electrostatic nature (Gospodarowicz et al.,1987a).



Sulfate ions have also been shown to bind to the sidechain equivalent of aFGF Lys-113 in the crystal structure of bFGF (Eriksson et al., 1991). There are at least 24 basic residues exposed on the surface of bFGF, five of which are conserved between bFGF and aFGF and form a prominent cluster within the carboxyl-terminal repeat of the molecule (ie., residues 119, 120, 125, 129 and 135). The quaternary amino groups of lysines -119, -125 and -129 form a nearly equilateral triangle and there are 2 ordered sulfate ions, one forming ionic contacts with Lys-119 and Lys-129 and the second linking Arg-120 and Lys-125 which are thought to mimic HS moieties. A contour map of the electrostatic potential surface shows the most prominent centre of positive charge to correspond to this group of conserved lysines and arginines, and to be located adjacent to the putative receptor-binding loop (residues 106 to 115) (Zhang et al., 1991).

### **1.7.3. Nature of the Heparin/Heparan Sulfate Motif which Interacts with FGFs**

In addition to binding to FGF-receptors on the cell surface, acidic and basic FGFs bind to HSPGs on the cell surface (Moscatelli 1987) and in the ECM (Vlodavsky et al., 1987a). HSPGs are sites of low affinity ( $K_d$  2-10nM) and of high capacity  $[(0.5-2) \times 10^6 \text{ sites/cell}]$  for acidic and basic FGFs (Moscatelli 1987). An important question to be answered is whether FGFs recognize a particular sugar sequence in heparin and HS, as does ATIII, and if so, is this a means by which HSPGs can regulate the interaction of FGFs with the FGF signaling receptor. Affinity coelectrophoresis of ATIII with heparin produces two distinct heparin subpopulations, as would be expected for ATIII binding to only a specific sequence on a portion of heparin molecules (Lee & Lander 1991). A similar result was seen with FN suggesting that the FN/heparin interaction may be due to a distinct GAG sequence. However, analysis of aFGF and bFGF showed no evidence for heparin subpopulations (Lee & Lander 1991), suggesting an extensive interaction of the FGFs with heparin molecules. In fact, there is a recent report demonstrating that 10-15 aFGF



molecules can bind to a single 16kDa heparin molecule. Such an observation implies one aFGF molecule for every 4-5 monosaccharide units on a single heparin chain (Mach et al.,1993).

Furthermore, when a wide range of polyanions were tested for their ability to stabilize hraFGF (15-154) against thermal denaturation, the interaction was found to be one of very low specificity with a surprising number of polyanions being able to induce physical stability (Volkin et al.,1993). Low molecular weight heparin, sulodexide, dextran sulfate, fucoidan and pentosan polysulfate all stabilized aFGF well and even ATP, inorganic phosphates, phosphorylated inositols, polynucleotides and inositol hexasulfate were effective. Similar results were obtained with aFGF potentiation (Belford et al.,1993). The common feature of all these compounds is the presence of one or more regions of high negative charge density such as regions of sulfation or phosphorylation.

Despite these studies suggesting the relatively non-specific binding of polyanions to FGF, recent studies indicate that FGF binds to a particular heparin/HS motif (see Table 1.11). Turnbull et al.,(1992) used bFGF affinity chromatography to examine the structural properties of fibroblast HS that enable it to bind bFGF with high affinity. They isolated an oligosaccharide component from the parent HS molecule, seven disaccharides in length, which binds bFGF with the same affinity as undegraded HS. Their data suggest that contiguous sequences of IdoA(2-OSO<sub>3</sub>) $\alpha$ 1,4 GlcNSO<sub>3</sub> are important in mediating high affinity binding between fibroblast HS and bFGF. The 2-O sulfate of IdoA (2-OSO<sub>3</sub>) and the N-sulfate of GlcNSO<sub>3</sub> appear to be essential for strong binding to bFGF.

Habuchi et al.,(1992) also identified a HS oligosaccharide capable of binding to bFGF. In agreement with Turnbull et al.,(1992), Habuchi et al.,(1992) suggest



Table 1.11:  
Saccharide Sequences of Heparin/Heparan Sulfate Implicated in FGF Binding

Predominant disaccharide	Fragment size	Reference
<u>Basic FGF</u>		
IdoA(2-OSO <sub>3</sub> )α1,4 GlcNSO <sub>3</sub>	tetradecasaccharide	Turnbull et al., (1992)
IdoA(2-OSO <sub>3</sub> )α1,4 GlcNSO <sub>3</sub>	octasaccharide	Habuchi et al., (1992)
IdoA(2-OSO <sub>3</sub> )α1,4 GlcNSO <sub>3</sub> (6-OSO <sub>3</sub> )	hexasaccharide	Tyrrell et al., (1993)
<u>Acidic FGF</u>		
IdoA(2-OSO <sub>3</sub> )α1,4 GlcNSO <sub>3</sub> (6-OSO <sub>3</sub> )	tetrasaccharide	Mach et al., (1993)
IdoA(2-OSO <sub>3</sub> )α1,4 GlcNSO <sub>3</sub> (6-OSO <sub>3</sub> )	hexasaccharide	Barzu et al., (1989)

that binding to bFGF may be dependent upon specific sugar sequences and sulfation patterns in HS. While Turnbull et al., (1992) used heparan sulfate from human fibroblasts, Habuchi et al., (1992) used heparan sulfate from pig aorta and the EHS tumour. In their system, the smallest fragment which bound bFGF was an octasaccharide composed of clusters of three IdoA (O-OSO<sub>3</sub>)-GlcNSO<sub>3</sub> disaccharide units (Habuchi et al., 1992). The fraction of HS from pig aorta which had high affinity for bFGF had a molecular weight of 47kDa and they suggested that there may be one or two bFGF-binding sites on each HS chain. Heparin cofactor II has been shown to bind to a similar cluster of units, namely IdoA(SO<sub>4</sub>) GalNAc(4SO<sub>4</sub>) units in skin dermatan sulfate (Maimone & Tollefsen 1990). Therefore, it is proposed that clusters of IdoA(2SO<sub>4</sub>)-containing disaccharide units may be important in controlling the biological activity of HS and dermatan sulfate and that they may be regulated by the same biosynthetic mechanisms (Bame et al., 1991; Fransson 1987; Lindahl et al., 1986; Gallagher et al., 1986).

The observation that the ability of a HS fraction to protect aFGF from proteolytic inactivation corresponded to its ability to bind bFGF, suggests that aFGF and bFGF may recognize similar sugar sequences in HS (Habuchi et al., 1992). Indeed, oligosaccharides comprising IdoA(2-OSO<sub>3</sub>) $\alpha$ 1,4GlcNSO<sub>3</sub>(6-OSO<sub>3</sub>) bind, stabilize and potentiate aFGF activity (Barzu et al., 1989; Mach et al., 1993; Volkin et al., 1993) (see Table 1.11). It is not yet clear, however, whether the 6-OSO<sub>3</sub> is relevant to the interaction between aFGF and heparin/HS.

On the other hand, a recent study by Nurcombe et al., (1993) clearly demonstrated that during murine neural cell development, aFGF and bFGF preferentially bind HSPG species which are differentially glycosylated. HSPGs derived from E9 preparations were found to be over 4-fold more effective in binding bFGF than aFGF, while HSPGs from E11 neuroepithelium bound aFGF



6-fold more effectively than bFGF. Examination of the HSPGs from E9 and E11 indicated a single, unique species of HSPG which differed only in the size and number of their GAG chains. HSPG isolated from E9-conditioned medium had an average GAG chain of approximately 20kDa and approximately 20 side chains attached per core protein synthesized, and those from E11 had GAG side chains of 35kDa with approximately 12 side chains attached per HSPG core protein. The specificity of the interaction between the two growth factors and the HSPGs appears to be the result of differential glycosylation of the same protein core at the two ages.

#### **1.7.4. Functional Relevance of Heparin/ Heparan Sulfate Binding by FGF**

Heparin potentiates the biological activity of aFGF, but generally not that of bFGF (Thornton et al., 1983; Orlidge & D'Amore 1986; Schreiber et al., 1985), by as much as 100-fold *in vitro* (Thornton et al., 1983). It is not clear whether such an enhancement of aFGF activity occurs *in vivo*, but the results of Kessler et al., (1976) show a correlation between the density of heparin-containing mast cells and neovascularization. It is therefore possible that mast cells release heparin at sites of neovascularization to stimulate the angiogenic activity of aFGF. The mechanism by which heparin potentiates the biological activity of aFGF is not fully understood but it is generally thought that the binding affinity of the particular heparin species for the growth factor correlates with its potentiating ability. However, the results of Belford et al., (1992) show that a carboxyl-reduced form of heparin which has the same binding affinity for aFGF as that of native heparin, is less potent at augmenting aFGF-induced mitogenesis. These results suggest that mechanisms other than those based entirely on a heparin-aFGF interaction, such as one involving the direct action of heparin on cell surface heparin-specific receptors, may be required for the potentiation of aFGF activity by heparin (Belford et al., 1992).



Several models which are not necessarily exclusive, have been proposed to explain the biological relevance of heparin/HS binding by acidic and basic FGF.

#### **1.7.4.1. Secretion Model**

Since FGFs lack a signal sequence, it has been suggested that bFGF may form an intracellular complex with HS found in the cytoplasm and nucleus (Ishihara et al., 1987) which is then inserted into the cell surface or deposited in the ECM. However, since HSPG synthesis occurs in the Golgi complex and exocytosis is via membrane-bound vesicles, it is unlikely that FGF would come into contact with the HSPG.

#### **1.7.4.2. Protection from Proteolysis Model**

Both basic and acidic FGFs are very sensitive to proteases, which may be due to their high arginine and lysine content. Proteases generated in areas of neovascularization may therefore inactivate them, and one important role of HSPG in the ECM and on the cell surface may be to protect FGFs from proteolytic digestion by plasminogen activators and collagenase. An example is the ability of heparin to protect aFGF against cleavage by thrombin. After incubation of aFGF with 50U/ml of human thrombin for 1 hour at 37°C, aFGF is degraded and is at least 50-fold less potent at stimulating mitogenesis than the uncleaved mitogen (Lobb 1988b). Heparin at 50ug/ml is able to protect aFGF against cleavage by thrombin. Interestingly, bFGF exhibits negligible degradation and its mitogenic activity is unchanged after incubation with thrombin for 6 hours (Lobb 1988b). In addition, in the absence of heparin, FGFs particularly aFGF, are relatively unstable at physiological temperature and at low pH. Heparin and a variety of sulfated polysaccharides have been shown to stabilize aFGF by binding to the native conformation of the protein (Gospodarowicz & Cheng 1986). The relatively low specificity required suggests that molecules other than heparan sulfates may also be able to stabilize the growth factors *in vivo* (Copeland et al., 1991). Although protection



from proteolysis is likely to be a component of the mechanism of potentiation of aFGF by heparin, it does not account for all the potentiation which can be as great as 100-fold (Damon et al., 1988 & chapter 3).

#### **1.7.4.3. Reservoir Model**

Cell surface HSPGs appear to provide a slow-release reservoir of FGF thereby allowing for long term responses after only brief exposure of the cells and ECM to bFGF (Flaumenhaft et al., 1989). In terms of potentiation of aFGF by heparin, aFGF may be concentrated on the cell surface in greater quantities when complexed to heparin which binds to a large number of cell surface receptors specific for heparin. This would increase the amount of aFGF available to interact with the FGF receptor. Furthermore, acidic and basic FGFs are thought to be deposited into the ECM of vascular or corneal cells (Vlodavsky et al., 1991c; Schweigerer et al., 1987c) where they bind to HSPGs (Jeanny et al., 1987; Folkman et al., 1988) and are protected from proteolytic degradation and inactivation. It is proposed that while the ECM would provide a source of FGF which could be utilized during localized endothelial cell proliferation and neovascularization, it would also sequester FGFs preventing them from acting on the vascular endothelium and thus maintaining a very low rate of endothelial cell turnover and vessel growth (Vlodavsky et al., 1991a,b).

This model requires a mechanism for the selective release of FGF from the ECM and heparanase activity has been implicated. Basic FGF may be released from basement membranes and ECMs as a noncovalent complex with a HS fragment as a result of heparanase activity provided by platelets, neutrophils, and tumour cells when they attach to the subendothelium (Matzner et al., 1985). Furthermore, bFGF complexed to HSPG stimulates the production of plasminogen activator by endothelial cells (Saksela & Rifkin 1990) which generates plasmin and may ultimately lead to further release of bFGF from the ECM as may plasminogen activator stimulation of heparanase activity (Bar-Ner



et al.,1986). It is unlikely that FGF would be released, not complexed with HSPG as it would probably be quickly inactivated by proteinases. On the other hand, enzymatic release of FGF from a HS complex might provide a mechanism for the rapid termination of its biological activity (Copeland et al.,1991).

#### **1.7.4.4. Receptor Binding Model**

Evidence is rapidly accumulating which indicates that the binding of FGF to heparin or to HSPG is an essential requirement for activation of the FGF receptor by FGFs. This has been demonstrated for the binding of bFGF to the high affinity Flg/FGFR1 receptor by Yayon et al.,(1991) where CHO cells lacking HSPGs, and transfected with the FGFR1 receptor, were only able to bind bFGF in the presence of heparin. Rapraeger et al.,(1991) and Olwin & Rapraeger (1992) also showed that treatment of various cells with heparitinase or culturing of cells in sodium chlorate which blocks sulfation, caused a drastic reduction in the binding of aFGF, bFGF and Kaposi's sarcoma FGF to their receptors and that binding could be restored by addition of heparin. Similarly, when murine FGFR2 was transfected into receptor-negative CHO cells and 32D myeloid cells, exogenous heparin was required for bFGF and Kaposi sarcoma FGF to bind FGFR2 and to induce proliferation in 32D cells lacking HSPGs. In contrast, when FGFR2 was introduced into CHO cells that express HSPGs, binding and activation of FGFs occurred in the absence of exogenous heparin, indicating that endogenous HSPG and exogenous heparin can act interchangeably. Similarly, when receptor-negative FDC-P1 cells were transfected with vectors containing FGFR1 with the 3 Ig-like and 2 Ig-like domains, the cells bind aFGF and bFGF but only in the presence of heparin (Bernard et al.,1991). In a related study, Ornitz and Leder (1992) demonstrated that with soluble murine FGFR1 binding of acidic and basic FGF was totally heparin dependent, whereas with soluble FGFR3, aFGF did exhibit some affinity for soluble FGFR3 in the absence of heparin but its binding affinity



was markedly increased in the presence of heparin. Ornitz and Leder (1992) suggested that this aFGF binding, in the absence of heparin, may not represent a functional high affinity complex and moreover, an FGFR3-mediated mitogenic response to aFGF exhibited an absolute requirement for heparin.

### **1.8. Role of HSPGs in FGF receptor binding**

As outlined above, there is now considerable evidence indicating that heparin/HS binding is essential for acidic and basic FGF to interact effectively with FGF receptors. Hitherto, three models have been proposed, which take this requirement for HSPG interaction into consideration, namely a conformational change model, a ternary complex model and a dimerization model. Each of these will models will be discussed briefly below.

#### **1.8.1. Conformational Change Model**

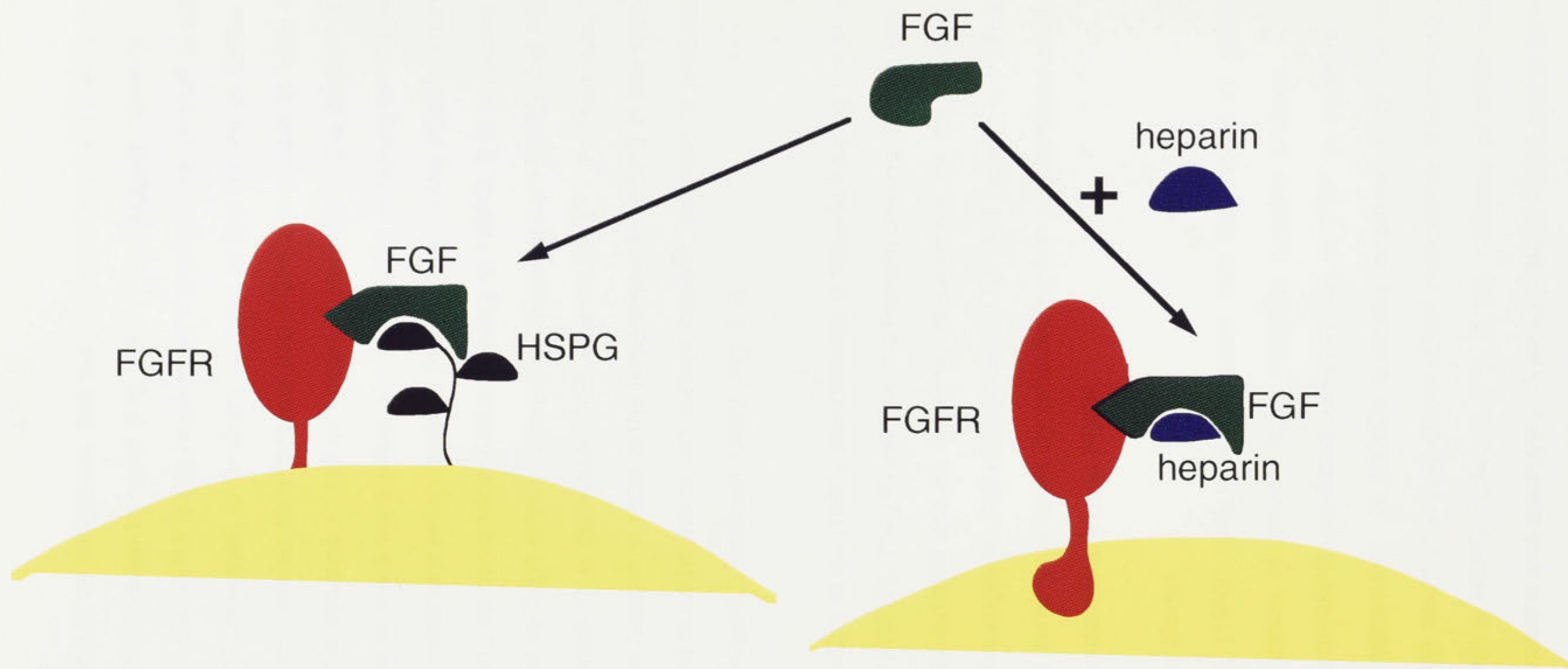
Yayon et al.,(1991) suggest that interaction of HSPG or heparin with bFGF produces a conformational change in bFGF which allows it to interact with the bFGF receptor (Fig. 1.8). In support of the conformational change model, Rapraeger et al.,(1991) suggest that the intrinsic affinity of the FGF receptor for bFGF is low, but that a complex of FGF, receptor and HSPG results in a high affinity interaction. They also propose that FGF undergoes a conformational change after interaction with HSPG. Similarly, Ornitz et al.,(1992) suggest that bFGF, its receptor and heparin/HSPG form a stable trimolecular complex possibly as a result of bFGFs change in conformation after its initial interaction with heparin or HS. Heparin has also been proposed to potentiate aFGF activity by producing a conformational change in the molecule which enhances its affinity for FGFRs.

#### **1.8.2. Ternary Complex Model**

Analysis of the kinetics of the association and dissociation of bFGF from its receptor has led Nugent & Edelman (1992) to propose that bFGF has as low an affinity for FGFR as it does for HSPG and it is not until a ternary complex is

FIGURE 1.8: An induced-fit model for heparin-dependent high affinity receptor binding of bFGF. It is proposed that in the absence of heparin, bFGF exists in a conformation that is not able to interact with its receptor. Interaction with heparin in solution or HSPG on the cell surface convert bFGF to a conformation that is recognized by the receptor. (From Yayon et al., 1991)





Induced Fit Model  
*Yayon et al (1991)*

formed between the three molecules, that the interaction is of sufficiently high affinity for receptor activation to occur (Fig. 1.9). They suggest, however, that binding of bFGF to the HSPG is not required as a prerequisite for FGF binding to the FGFR. This, however, is in direct contrast to the results of Ornitz et al., (1992) and others whose data indicate that FGFs are unable to bind the FGF-receptor in the absence of HS or heparin. Perhaps in the Nugent and Edelman experiments, there were some residual HSPGs remaining on the cells after heparitinase treatment which enabled FGF to bind to the FGFR. Rather than bFGF changing its conformation after binding to HSPG, it is proposed to undergo a cooperative "high affinity" interaction requiring both HSPG and FGF-receptor which results in a decreased "off" rate for bFGF. Furthermore, for exogenously added heparin to replace the requirement for HSPG in this model, heparin would need to be bound to cell surface heparin receptors to produce a ternary complex (Nugent & Edelman 1992) (Fig. 1.9).

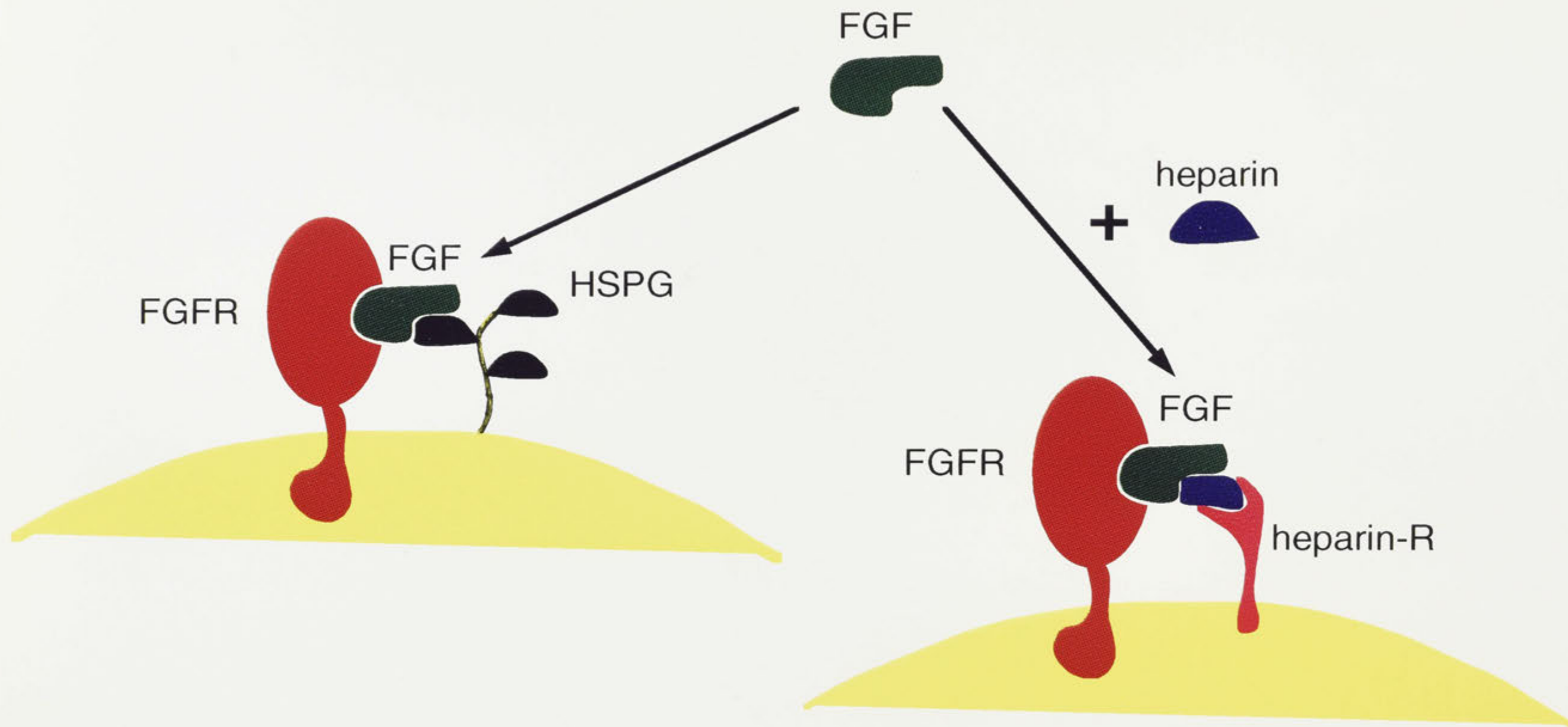
Recent studies by Kan et al., (1993) demonstrate a heparin binding site in the Ig-like domain II of FGFR1. They found that this heparin binding domain and its associated heparan sulfates are an essential requirement for the binding of FGF to FGFR1. This suggests that complexes of FGF and heparin or FGF and HSPG may form a ternary complex by crosslinking the growth factor-binding and heparin-binding domains on one FGFR.

### **1.8.3. Dimerization Model**

Mascarelli et al., 's (1993) data are consistent with the model of general allosteric oligomerization of growth factor receptor tyrosine kinases. They propose that heparin/HSPG mediated dimerization of FGF is essential for productive FGFR interaction. A 150kDa complex, observed in bovine lens epithelial cells, is the result of a very stable aFGF homodimer crosslinking an FGFR molecule with a HSPG molecule (Fig. 1.10). Dimers of bFGF have been observed in neurons and astrocytes (Walicke & Baird 1991) and in retinal



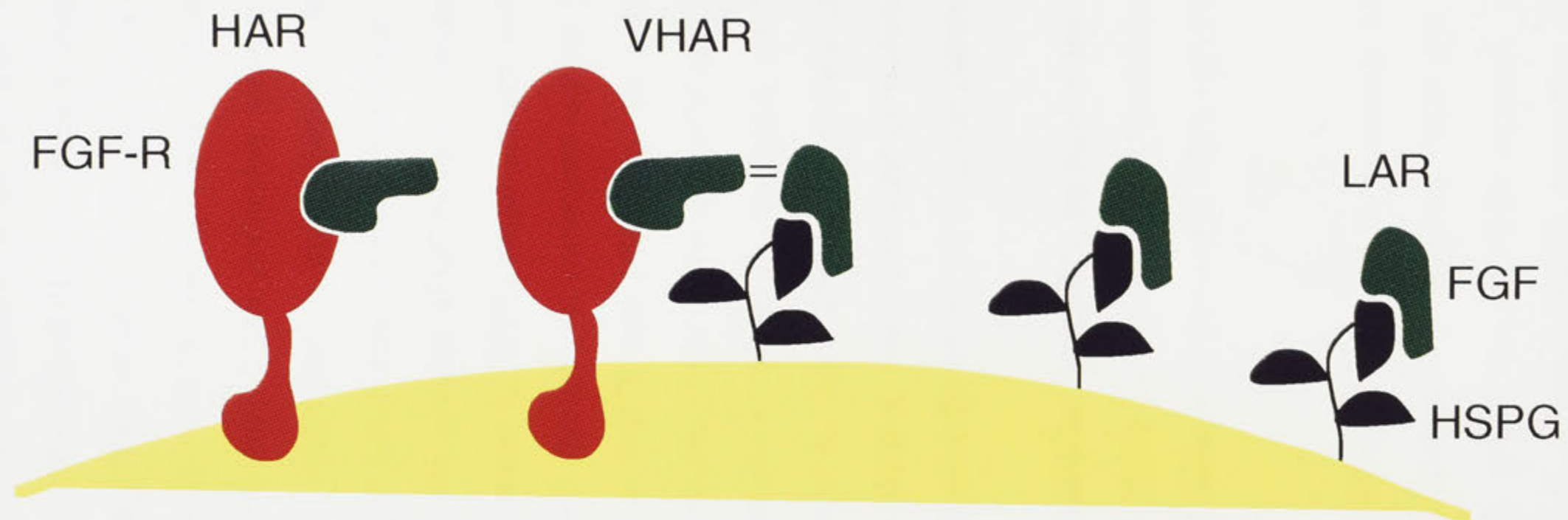
FIGURE 1.9: Ternary complex model for "high affinity" bFGF binding. "High affinity" binding is represented as a ternary complex with bFGF bound simultaneously to FGFR and HSPG. In the absence of HSPGs, a ternary complex forms in which bFGF and soluble heparin crosslink FGFR and a heparin receptor. (From Nugent & Edelman 1992). In this model the heparin receptor is depicted as a separate molecule on the cell surface but it could represent a heparin binding site on the FGFR as suggested by Kan et al.,(1993).



Ternary Complex Model  
*Nugent and Edelman (1992)*



FIGURE 1.10: Dimerization model for aFGF high affinity binding. It is proposed that monomeric and oligomeric aFGF may bind to HSPG, the low affinity receptor (LAR). Monomeric aFGF may bind to FGF-R, the high affinity receptor (HAR). Dimerization of aFGF leads to interaction of HAR with LAR forming the very high affinity receptor (VHAR). (From Mascarelli et al., 1993)



Dimerization Model  
*Mascarelli et al (1993)*



pigmental epithelial cells. Ornitz et al.,(1992) have shown that dimerization of bFGF in solution can be induced in the presence of heparin or by heparin oligosaccharides 6, 8 and 16 sugar residues in length but that optimal dimer formation is highly dependent on the ratio of aFGF to heparin oligosaccharide. Mach et al.,(1993) suggest that dimers of aFGF could form as a result of the large number of aFGF molecules (10-15) able to bind to a single 16kDa heparin molecule. Ligand dimerization has previously been shown to be involved in either inducing or stabilizing receptor dimers. Mascarelli et al.,(1993) suggest that dimers of aFGF could form as the result of an aFGF molecule binding to the FGFR, and another aFGF molecule binding to HSPG. Alternatively, an aFGF dimer bound to HSPG could interact with an FGFR, producing an aFGF-dimer-FGFR complex of molecular weight 150kDa. In either case, the crosslinking of FGFR and HSPG by an aFGF dimer is proposed to result in a greater affinity of the FGFR for aFGF and this complex has been termed the "very high affinity receptor". In support of this idea that dimerization of FGF is important for high affinity cell surface binding, high concentrations of heparin were found to inhibit formation of multimers of aFGF (Mascarelli et al.,1993) and displace high affinity binding complexes.

### **1.9. Transmembrane and Intracellular Signalling by Acidic and Basic FGF**

In this section I will highlight some of the important transmembrane and intracellular signalling events following engagement of FGFs with FGFRs.

#### **1.9.1. Receptor Dimerization**

Although FGF-dependent signalling is initiated immediately following the binding of FGF to its receptor (Johnson & Williams 1993) the intracellular signalling mechanisms which give rise to FGF-induced proliferation, differentiation or maintenance of a differentiated phenotype are not well understood. Similar to other growth factor receptors (Williams 1989; Ullrich & Schlessinger 1990), FGFR dimerization is induced upon binding of aFGF or



bFGF (Bellot et al.,1991; Ueno et al.,1992) and both homodimeric and heterodimeric receptor species involving FGFR1, FGFR2 and FGFR3 (Bellot et al.,1991; Ueno et al.,1992) have been observed. Homodimers of FGFR are thought to be required for signal transduction, although, it may be possible that in cells expressing more than one type of FGFR, signalling may occur through heterodimers as well as through homodimers. Heterodimers may activate cytoplasmic signalling pathways differently than do homodimers (Ueno et al.,1991). Oligomerization of receptors may be induced by monomeric ligands which induce conformational changes (Greenfield et al.,1989) resulting in receptor-receptor interactions, or by bivalent ligands that mediate dimerization of neighbouring receptors (Seifert et al.,1989; Heldin et al.,1989; Hammacher et al.,1989) (see section above). Receptor oligomerization produces a conformational change in the extracellular domains, resulting in increased and more stable interactions between cytoplasmic domains, and leads to receptor autophosphorylation (Huang & Huang 1986; Coughlin et al.,1988; Mansukhani et al.,1990) and ultimately to elevated protein tyrosine kinase activity,

### **1.9.2. Intracellular Messengers Implicated in FGF Activity**

As already mentioned, the intracellular signalling mechanisms induced by FGF binding to its receptor are not well understood and there are many contradictions in the literature as to whether G-protein mediated signalling pathways are involved in FGF-induced DNA synthesis (see Table 1.12). When used alone on hamster lung fibroblast cells, bFGF does not appear to utilize any of the major G-protein-mediated signalling pathways and its actions are thought to be primarily mediated by the intrinsic protein tyrosine kinase of its receptors (Paris & Pouyssegur 1991). However, these results are in direct contrast to those of Logan & Logan (1991) which indicate that pertussis toxin is a potent inhibitor of bFGF-induced DNA synthesis implying that G-proteins do have a role in the mitogenic pathways for bFGF. The substrate for pertussis toxin is  $G_i$  which modulates adenylate cyclase activity, and pertussis toxin



Table 1.12:  
Intracellular Messengers Implicated in FGF-induced DNA Synthesis

Cell type	Messenger	Reference
Hamster lung fibroblasts	protein tyrosine-kinase activity, no G-protein mediated signaling	Paris & Pouyssegur (1991)
	Gi couples adenylate cyclase to bFGF-receptor Gp	Logan & Logan (1991)
3T3 fibroblasts	increased intracellular pH	Halperin & Lobb (1987)
	increased calcium levels	Tsuda et al.,(1985)
	increased phosphorylation of PLC $\gamma$	Burgess et al.,(1990a)
	increased hydrolysis of phosphoinositides	Brown et al.,(1989)
	phosphorylation of MAP-2 kinase	Rybak et al.,(1988)
	phosphorylation of raf	Rapp et al.,(1991) Friesel et al.,(1989)
	phosphorylation of p60, p85, p90, p130	Zhan et al.,(1993)
	increased transcription of c-fos, c-myc and c-jun	Burgess et al.,(1990b)
	protein kinase C activation	Logan & Logan (1991)
	inhibition of adenylate cyclase activity	
L6-myoblasts	activation of tyrosine kinase by PKC-independent phosphorylation	Lee et al.,(1989)
	translocation to the nucleus	Bouche et al.,(1987)
	calcium mobilization	Peters et al.,(1992b)
	phosphatidylinositol hydrolysis not required	Peters et al.,(1992b)



treatment inhibited a bFGF-induced fall in intracellular cAMP levels.  $G_i$  is therefore implicated in coupling adenylate cyclase to the bFGF receptor(s).  $G_p$ , linked to phospholipase C, may also be involved in the intracellular signalling pathways for bFGF (Logan & Logan 1991).

The intracellular messengers observed to be activated after stimulation of cells with acidic and basic FGF are listed in Table 1.12. However, it is yet to be determined which messengers are activated directly as a result of FGF stimulation and have a direct role in DNA synthesis. It is not known whether modulation of protein kinase C, cAMP and inositol phosphates occur as a result of direct coupling to the bFGF receptor(s), to the signalling pathways, or whether they occur secondary to activation of other mechanisms (Logan & Logan 1991). Nor is it understood what changes in signalling patterns allow FGFs to regulate cellular differentiation and chemotaxis, to influence cell-shape and to maintain cell survival without stimulating DNA synthesis. The cell types in which the messengers were observed to be activated are listed in Table 1.12 because although all cells listed undergo DNA synthesis in response to FGF, inconsistencies over which signals are required, seem to arise when different cell types are compared. Furthermore, some of the messengers activated may well be involved in differentiative responses. For example, a similar set of intracellular messengers is produced in neurons and yet these cells do not undergo cell division but survive and differentiate in response to FGFs. An example of this uncertainty is the controversy over the role of PLC- $\gamma$  in FGF receptor mediated signalling. PLC- $\gamma$  is found directly associated with the receptor (Mohammadi et al., 1991; Peters et al., 1992b) and Logan & Logan (1991) have demonstrated a correlation between FGF mitogenesis and production of inositol phosphates. This is in contrast to Peters et al., (1992b) who showed that L6 myoblasts, expressing a mutant receptor which does not associate with or phosphorylate PLC- $\gamma$  but does phosphorylate itself and several other proteins, are still able to proliferate in response to FGF (Peters et



al.,1992b). This result suggests that neither calcium mobilization nor phosphatidylinositol hydrolysis are required for FGF-induced mitogenesis in L6 myoblasts. Furthermore, FGF does not stimulate hydrolysis of phosphoinositides in Chinese hamster lung fibroblasts (Magnaldo et al.,1986) or only gives a relatively small response when compared to that observed with other growth factors (Peters et al.,1992b). These contradictory results have led to the suggestion that other signalling pathways must be required for FGF-induced mitogenesis and that maybe phosphatidylinositol hydrolysis is important in non-mitogenic cellular responses, such as, chemotaxis, cell-shape changes (Goldschmidt-Clermont et al.,1991) and in the regulation of cellular differentiation in early amphibian embryogenesis. However, there is some evidence to suggest that cell survival is less independent of phosphatidylinositol hydrolysis (Magnaldo et al.,1986; Chambard et al.,1987; Moenner et al.,1987).

Further controversy over which messengers are important in DNA synthesis occurs when FGF is used in combination with other factors. For example, FGF stimulation of mouse fibroblasts results in a fall in cAMP levels whereas when used in combination with prostaglandin E, forskolin or cholera toxin in hamster fibroblasts, cAMP accumulation is potentiated by up to 50% (Paris & Pouyssegur 1991). Since this potentiation is insensitive to pertussis toxin, FGF is thought to enhance activation of adenylate cyclase through Gs, the stimulatory G protein (Magnaldo et al.,1989) and not through Gi, the inhibitory G protein.

#### **1.10. Localization of FGF in the Nucleus**

Not only do FGFs stimulate intracellular messengers to transmit signals to the nucleus but there is increasing evidence to suggest that they may also act directly within the cell nucleus (Kardami & Fandrich 1989; Renko et al.,1990; Tessler & Neufeld 1990; Suzuki et al.,1991; Brigstock et al.,1991). Growth



factors may be localized to the nucleus to allow activation of pre-existing nuclear receptors resulting in protein kinase activity; or to enable direct interaction of growth factor with nuclear receptors or chromatin, ultimately leading to increased mRNA efflux, or enhanced chromatin condensation in preparation for mitosis (Burwen & Jones 1987).

It has been proposed that a complex of aFGF and HS could be internalized by FGF-receptor mediated endocytosis and targeted to the nucleus with the aid of the nuclear translocation sequence on aFGF. This would ensure that the growth factor was protected from proteolysis during transport to the nucleus (Habuchi et al., 1992). Indeed HS enriched in  $\text{GlcA}(2\text{SO}_4)\text{GlcNSO}_3(6\text{SO}_4)$  has been found in the nuclei of rat hepatoma cells (Fedarko & Conrad 1986). Rusnati et al., (1993) have also speculated that bFGF is internalized via HSPGs and transported to the nucleus as a complex.

However, the lack of evidence supporting the release of bFGF from cells has led to the proposal that endogenous bFGF may have a biological function within its cell of origin (Logan 1990). The observation that suramin, a molecule that inhibits the binding of bFGF to its plasma membrane receptor, did not effect the amount of biosynthetically labeled bFGF present in the nucleus suggests that the nuclear localization of endogenous bFGF is not the result of receptor-mediated uptake of released bFGF (Dell'Era et al., 1991).

It is proposed that FGF is transported into the nucleus by a nuclear targeting signal (Imamura et al., 1990; Bugler et al., 1991; Quarto et al., 1991; Florkiewicz et al., 1991b) where it directly regulates the transcription of specific genes. It appears not to recognize distinct DNA regions but binds to the DNA in a nonspecific manner. Brigstock et al., (1991) found that bFGF could be displaced from chromatin by heparin, suggesting an intra-nuclear interaction between bFGF and heparin-like molecules may occur. Alternatively, a heparin-



sensitive association between bFGF and other anionic substances such as DNA is possible. However, preliminary results of Amalric et al.,(1991) indicate that bFGF may bind DNA with nucleotide sequence specificity.

The intracellular localization of bFGF is tightly regulated during the cell cycle with bFGF being detected in the nucleus during the final two hours of the G1 phase (Hill & Logan 1992; Baldin et al.,1990). Basic FGF's presence in the nucleolus is accompanied by the activation of ribosomal RNA transcription and *in vitro*, by stimulation of pre-rRNA synthesis (Bouche et al.,1987), a major event in the transition from a quiescent state to full proliferation (Pardee et al.,1985). Basic FGF also increases the transcriptional activity of RNA polymerase 1 in nuclei isolated from quiescent sparse adult bovine aortic endothelial cells by a factor of 5.6 (Bouche et al.,1987).

Basic FGF has also been shown to inhibit the transcription of Pgk-1 and stimulate the transcription of Pgk-2, two spermatogenic genes encoding phosphoglycerate kinases (Nakanishi et al.,1992). Basic FGF may compete with trans-acting factors for binding to corresponding cis elements and have either a stimulatory or inhibitory effect on transcription depending on the mode of action of the cis elements (Nakanishi et al.,1992) .

The establishment of mesodermal and endodermal cell lineages during *Xenopus* embryogenesis has been proposed to depend upon the action of hormones and growth factors in the nucleus (Burwen & Jones 1987; Logan 1990) and the results of Shiurba et al.,(1991) support the idea that FGFs may act directly on the genome during embryonic induction. Shiurba et al.,(1991) have monitored the spatial localization of aFGF and bFGF within the *Xenopus laevis* embryo at successive developmental stages. Some FGFs appeared to be prepositioned within the egg in association with yolk in the vegetal hemisphere where they remain during cleavage. However, at the time of



mesoderm induction, they shift from the cytoplasm to the nucleus in cells of the marginal zone. Although the data provide considerable evidence for the translocation of FGFs to the nucleus, the functional relevance of their nuclear localization remains to be determined.

### **1.11. Aims of the Present Study**

The importance of HSPGs in FGF action is only now beginning to be appreciated. Acidic and basic FGF are distributed widely throughout the body and yet they must remain inactive for much of the time. It is becoming increasingly clear that HSPGs play a major role in regulating FGF activity. Thus, the interaction between FGF and cell surface HSPG is now known to be essential to the central event in growth factor action, that is, the binding of the growth factor to the signal transducing receptor. HS chains may also regulate FGF activity by varying the affinity of the interaction between FGF and HSPG. This was shown to be the case by Nurcombe et al.,(1993) who showed that during murine neural precursor cell development, acidic and basic FGF activity was differentially modulated in part, by variations in the glycosylation of a particular HSPG. In addition, HSPGs sequester FGFs and store them in a stable and protected form in the ECM. Heparin is also able to modulate many of the biological activities of the FGFs in an apparently similar manner to HSPGs. However, the mitogenic activity of aFGF is potentiated by heparin and although not generally regarded as a physiological phenomenon, an understanding of the mechanism involved, would further our understanding of the requirements for FGF activation and the role of heparin-like molecules in this process.

An understanding of the requirements for the interaction between FGFs and HS chains, and of how acidic and basic FGF activity is modulated by HSPGs, may facilitate the development of compounds which disrupt this interaction thereby inhibiting the interaction of FGF with its signaling receptors. Such inhibitors of



FGF action could be important in the inhibition of angiogenesis which becomes a pathological process during tumour formation and many other diseases.

The major aims of the research presented in this thesis have been to try and understand why bFGF is more potent than aFGF *in vitro*, why the mitogenic activity of aFGF in cell culture is potentiated by heparin while that of bFGF is not, and the mechanism by which this potentiation occurs. Comparative studies were performed with both acidic and basic FGF in an attempt to further understand these phenomena and the possible involvement of heparan sulfates. These studies are described in the first two experimental chapters. In particular, the hypothesis that heparin enhances aFGF activity by forming a complex with aFGF which crosslinks both the FGFR and a receptor specific for heparin on the cell surface was examined. These studies also examined the intriguing observation that CR-heparin, although binding to aFGF as well as native heparin, was less effective at enhancing aFGF mitogenicity (Belford et al., 1992). All of these studies were performed with the long term aim of inhibiting FGF activity by disrupting the interaction of FGF with HSPG, and the third experimental chapter describes experiments specifically directed towards this goal. In this chapter, the effect of two naturally occurring heparin/HS binding proteins on FGF action is described.



## Chapter 2

### Materials and Methods

#### 2.1 Growth Factors and Heparin Binding Proteins

Human recombinant acidic and basic fibroblast growth factors were obtained from Bio Source International (Camarillo, CA) and Pepro Tech Inc. (Rocky Hill, NJ). Acidic and basic FGFs were reconstituted at 200ug/ml in PBS/0.1% CHAPS to prevent adherence to the walls of the tubes and aliquots stored in polyethylene tubes (Kartell, Milan, Italy) at -70°C. Aliquots were not frozen more than once and were not used any longer than two weeks after thawing.  $^{125}\text{I}$ -aFGF(1234Ci/mmol) and  $^{125}\text{I}$ -bFGF (920Ci/mmol) were obtained from Amersham International plc, Amersham, UK.  $^{125}\text{I}$ -aFGF was resuspended in distilled water to a final concentration of 600ng/ml and aliquots frozen.  $^{125}\text{I}$ -bFGF was resuspended in distilled water to give a final concentration of 200ng/ml and aliquots frozen.

Human antithrombin III (ATIII) and human platelet factor 4 (PF4) were obtained from Sigma Chemical Co, St Louis, Mo. ATIII was reconstituted in distilled water to give a final concentration of 143ug/ml. PF4 was desalted and resuspended in PBS to give a final concentration of 100ug/ml. Chicken and human histidine-rich glycoprotein (HRG) were purified by the method of Rylatt et al.,(1981).

#### 2.2. Polysaccharides

Heparin (bovine lung), HS (bovine kidney), HS-fast moving fraction (bovine intestinal mucosa), HS (bovine intestinal mucosa), hyaluronic acid (human umbilical cord), chondroitin-4-sulfate (whale cartilage), chondroitin-6-sulfate (whale cartilage), chondroitin-4,5-disulfate (whale cartilage), dermatan sulfate (porcine skin) and keratan sulfate (bovine cornea) were all obtained from Sigma Chemical Co, St Louis, Mo. Sodium sucrose octasulfate was a



generous gift from Bukh meditec A/S, Denmark. Heparan sulfate (porcine mucosal, 32kDa) was a generous gift from Organon International bv, Oss, Netherlands.

CR-heparin was prepared according to the method of Taylor et al.,(1976) and the number of carboxyl groups remaining on the various preparations of CR-heparin, relative to the number present on native heparin, was determined by the method of Bitter & Muir (1962). The ability of these preparations to compete for the binding of aFGF to heparin-agarose (Bio-Rad, Richmond, CA) was determined as described by Belford et al.,(1992). The following modified heparins: N-desulfated heparin; N-desulfated acetylated heparin; N-desulfated acetoacetylated heparin; N- & O-desulfated heparin and N- & O-desulfated, N-resulfated heparin, were provided by Dr C. Parish and prepared as previously described by Belford et al.,(1992).

### **2.3. Fluoresceination and Iodination of Heparins**

Heparin and CR-heparin were fluoresceinated by cyanogen bromide activation and conjugation with fluoresceinamine as previously described (Glabe et al., 1983). The concentration of the fluoresceinated heparin was estimated by N-de-sulfation and deamination by nitrous acid and the resulting anhydromannose estimated by indole-hydrochloric acid reagent, as described by Chandrasekaran & BeMiller (1980). The concentration of native heparin was estimated as 6.5mg/ml and the concentration of CR-heparin as 5.4mg/ml.

The fluoresceinated heparins were then iodinated by adding 3ul of the heparin in PBS (20ug of native heparin and 16ug of CR-heparin) to 13.7ul normal saline, 1.5ul of 0.2M borate buffer pH 8.0 and 5ul iodine-125 (500uCi) (Iodine-125, carrier free 100mCi/ml, Amersham) in an eppendorf tube and transferring the mixture to a small glass tube in which 20ul of 1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycouril (Iodogen; Sigma), at 0.26mg/ml in chloroform, had been dried



under  $N_2$ . The reaction mixture was incubated for 30 min on ice with regular mixing and the reaction was terminated by dilution of sample to 0.25ml with PBS. 5ul of sample was removed for counting. The sample was applied to a NAP-5 Sephadex G-25, DNA grade column (Pharmacia, Sydney, Australia) preequilibrated with PBS and eight fractions of 0.25ml collected and 5ul of each counted. The 3rd, 4th and 5th fractions were generally found to contain the majority of cpm and were pooled and stored at 4°C. Specific activity was approximately 18000 cpm/ng.

#### **2.4. Iodination of cHRG**

Chicken HRG (cHRG; 20ug) in a volume of 10ul was mixed with 50ul borate saline pH 8.0, and 4ul  $^{125}I$  (200uCi), before being transferred to a small glass tube containing 20ul Iodogen (0.26mg/ml) dried under  $N_2$ . Incubation was for 30min on ice with regular mixing. The reaction was terminated by the addition of 436ul PBS and a 5ul sample removed for counting. The sample was applied to a NAP-5 column, preequilibrated in PBS, and eluted in 1ml PBS. The specific activity was determined as  $3 \times 10^6$  cpm/ug HRG.

#### **2.5. Effect of Heparins on Digestion of aFGF by Trypsin**

To 12.5ul of each heparin preparation (1mg/ml in PBS/0.1% BSA) was added 12.5ul PBS/0.1% BSA, followed by 2ul  $^{125}I$ -aFGF (1.8ng). Controls received PBS/BSA in place of heparin. After mixing samples, 25ul trypsin (200ug/ml) (Cooper Biomedical, Glendale, CA) dissolved in 0.1M ammonium bicarbonate buffer pH 8, or 25ul of ammonium bicarbonate buffer pH 8 for controls, was added and samples left to react at 37°C. After 3 hours, a further 5ug of trypsin was added to each sample and samples left to react at 37°C, overnight. Samples of each treatment were then boiled for 5 min in reducing SDS-PAGE sample buffer, applied to an 11% SDS-polyacrylamide gel and electrophoresed at a constant current of 20mA for 2.5 hours. After fixation, the dried gel was



autoradiographed at  $-70^{\circ}\text{C}$  on Kodak XAR-5 film using a Cronex intensifying screen.

## 2.6. Cell Culturing

Mouse BALB/c 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD) supplemented with  $\text{NaHCO}_3$ , 1% L-glutamine, 20mM HEPES pH 7.4 and 10% fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia) at  $37^{\circ}\text{C}$  (5%  $\text{CO}_2$  incubator) in  $80\text{cm}^2$  tissue culture flasks (Nunc, Roskilde, Denmark). Cell monolayers were released for subculturing with 0.1% trypsin (Cytosystems, Sydney, Australia) and 0.1% EDTA in PBS when cells were subconfluent (every three days) and cells were then resuspended at  $1.5 \times 10^4$  cells/ml in culture medium. Confluent monolayers used in mitogenic and binding assays were prepared by seeding  $3 \times 10^3$  cells/well in 96-well plates (Nunc) in the same medium as used for subculturing, and incubating at  $37^{\circ}\text{C}$  for four to five days. For mitogenic assays, confluent monolayers were serum starved (DMEM/1% L-glutamine/20mM HEPES pH 7.4) for 48 hours.

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords by the method of Jaffe (1984) and cultured in Medium 199 (Gibco) supplemented with 20% FCS, 2% L-glutamine, antibiotics, 130ug/ml heparin and 1.2mg/ml of endothelial cell growth supplement (Sigma). They were grown in tissue culture flasks (Nunc), coated with 0.1% gelatin (Sigma) in distilled water. Cells generally reached confluence after seven days and were passaged at a density half that reached at confluence. The medium was replaced every four days. Only cells at passages two to seven were used in experimental work. When used in mitogenic assays, confluent monolayers of HUVE cells were released with 0.1% trypsin and 0.05% EDTA in PBS, and diluted in culture medium to a final concentration of  $7.6 \times 10^5$  cells/ml.



## 2.7. Heparinase Treatment of 3T3 Cells

In some experiments BALB/c 3T3 cells were treated with heparinase prior to  $^{125}\text{I}$ -FGF binding (Nugent & Edelman 1992). Heparinase 1 (EC 4.2.2.7; Sigma) in 350 unit lots was resuspended in 205ul of 200mM sodium acetate pH 7, 527ul of normal saline and 81ul of 2mg/ml BSA. Aliquots of 50ul were frozen on dry ice and ethanol before being stored at  $-70^{\circ}\text{C}$ . Confluent BALB/c 3T3 cells were washed once with  $37^{\circ}\text{C}$  binding buffer (DMEM, 1% L-glutamine, 20mM Hepes, 0.1% BSA pH 7.4) and treated with 50ul/well of heparinase, using 2 units/well for 30min at  $37^{\circ}\text{C}$  with  $^{125}\text{I}$ -aFGF and 4 units/well for 1 hour at  $37^{\circ}\text{C}$  with  $^{125}\text{I}$ -bFGF. These treatments generally resulted in the removal of 70-90% HSPGs as determined by comparing the number of cpm eluting from the cell surface of heparinase treated cells in 10 sec when washed with 2M NaCl in binding buffer with the number of cpm eluted by the same buffer from untreated cells. Heparinase and soluble degradation products were removed by washing the cells three times with ice cold binding buffer.

## 2.8. Isolation of HSPGs from 3T3 Fibroblasts

Heparan sulfate chains were isolated from BALB/c 3T3 fibroblasts according to the method of Turnbull & Gallagher (1991). Briefly, BALB/c 3T3 cells ( $19 \times 10^6$  cells) in Basal medium Eagle (BME, sulfate-free, Gibco)/10% FCS/1% L-glutamine/20mM Hepes pH 7.4 were seeded into  $6 \times 175\text{cm}^2$  flasks and grown to subconfluence in the presence of  $^{35}\text{SO}_4$  (50 uCi/ml) (25-40Ci/mg; Amersham). The conditioned media were collected and frozen for subsequent use. After treating cells with 2M NaCl for 30min to remove salt dissociable HSPGs, flasks were rocked in n-octyl- $\beta$ -D-glucopyranoside (octyl glucoside) (Sigma) for 30min at  $4^{\circ}\text{C}$  to dissociate detergent soluble HSPG from cell membranes. The octyl-glucoside supernatants were pooled and frozen. HS chains were isolated from conditioned media and octyl-glucoside supernatant as follows: The sample was applied to a DEAE-Sepharose column and GAGs eluted with 1M NaCl, 20mM phosphate buffer pH 6.8. Fractions containing the



most cpm were pooled, lyophilized and desalted on a PD-10 column. After digestion of HSPG core proteins with protease (5mg/ml) (from *Streptomyces griseus*; Sigma) overnight at 37°C, the sample was applied to a DEAE-Sepharose column and chondroitin sulfates eluted with 0.3M NaCl and heparan sulfates with 1M NaCl. The HS-containing fractions were desalted and digested with chondroitinase ABC (2mg/ml; EC 4.2.2.4; Sigma) for 6 hour at 37°C to remove contaminating chondroitins. The digest was applied to a DEAE-Sepharose column and the 1M NaCl eluate desalted, lyophilized and resuspended in 500ul of 10% ethanol in distilled water. Glucuronic acid determination showed that 17ug of HS was isolated from the conditioned media and 1ug HS from the octyl-glucoside lysate.

### **2.9. Binding of Radiolabeled Heparins to BALB/c 3T3 Cells**

Confluent cultures of BALB/c 3T3 cells in 96 well plates were washed twice with 200ul/well of binding medium (DMEM/ 0.5% BSA/ 20mM Hepes) whilst on ice. Serial dilutions of radiolabeled heparins (1.56-150ug/ml) were added in volumes of 100ul/well and nonspecific binding determined by including a 50-fold excess of unlabeled heparin in a replicate set of reaction mixtures. Following incubation for 5 hours on ice cell monolayers were washed once with 200ul/well of binding medium before being detached with 200ul/well 0.1% EDTA in PBS. After mixing with a multichannel pipette, cells were harvested with a Titertek cell harvester 530 (Flow Laboratories, Sydney, Australia) and counted by an Auto-gamma 5650 counter (Packard Instrument International, Meriden, CT). Scatchard analysis was performed using "Scatchard Plot" written by R.E. Viola.

In order to determine 3T3 cell number/well in binding assays, 5ul of a 1mM stock solution of Hoechst 33342 (Sigma) was added directly to each well and incubated for 15 min at 37°C. Medium containing the unbound dye was removed and the cells washed once with 200ul/well PBS. Cells were detached



by adding 100ul/well of 0.1% trypsin in PBS and incubated for 10min at 37°C. Detached cells were transferred to a 96-well flat bottomed microplate (Nunc) and their fluorescence determined by an automated fluorescence reader (Micro Fluor reader, Dynatech Laboratories Inc., Alexandria, VA).

In the case of inhibition assays, serial dilutions of unlabeled heparin and CR-heparin were made and 50ul of each concentration (0.04-5 mg/ml) added to triplicate wells. This was followed by the addition of 50ul/well of a constant concentration of either  $^{125}\text{I}$ -heparin or  $^{125}\text{I}$ -CR-heparin to give a final concentration of 100ug/ml. Following incubation on ice for 5 hours, cells were washed, detached and harvested as above.

#### **2.10. Binding of Radiolabeled Acidic and Basic FGF to BALB/c 3T3 Cells**

$^{125}\text{I}$ -FGF binding was conducted with confluent BALB/c 3T3 cells. Prior to the initiation of the  $^{125}\text{I}$ -FGF binding, the monolayers were washed once with 200ul/well of ice cold binding buffer (DMEM/1% L-glutamine/0.1% BSA/20mM Hepes pH 7.4) and then incubated at 4°C for 10min to precool the monolayers.

To determine dissociation rate constants for  $^{125}\text{I}$ -aFGF and  $^{125}\text{I}$ -bFGF, the method described by Nugent & Edelman (1992) was followed. Cells were incubated with  $^{125}\text{I}$ -bFGF (2ng/ml) or  $^{125}\text{I}$ -aFGF (10ng/ml) in binding buffer for 3 hours at 4°C to allow binding to reach equilibrium. When examining the effect of heparin on the rate at which aFGF and bFGF dissociated from FGFR, FGFs were preincubated with either heparin (0.1ug/ml) or medium for 1 hour at 4°C before being added to cells for the 3 hour incubation. The amount of  $^{125}\text{I}$ -FGF bound to nonspecific sites was determined by including an excess of the unlabelled growth factor (50ug/ml) in the buffer during the 3 hour incubation. After the incubation, the supernatant was aspirated and the monolayers washed three times with ice cold binding buffer before adding 100ul/well of dissociation medium and incubating the cells at 4°C for the required period of



time. For the dissociation kinetics of  $^{125}\text{I}$ -aFGF, the dissociation medium contained 1ug/ml of unlabeled aFGF and for bFGF dissociation kinetics, it contained 7ug/ml of unlabeled bFGF in binding buffer. Unlabeled FGF was included in the dissociation medium to ensure that released  $^{125}\text{I}$ -FGF would not rebind to unoccupied receptors (Nugent & Edelman 1992). A higher concentration of bFGF than aFGF was used because of bFGF's stronger tendency to reassociate with HSPG during the final washing process. After the dissociation period, the dissociation medium was removed, the cells washed once with ice cold binding buffer and the amount of  $^{125}\text{I}$ -aFGF or  $^{125}\text{I}$ -bFGF that remained bound to the HSPG released by exposing the cells to 2M NaCl in binding buffer for 10 sec. Radiolabeled FGFs bound to FGFR were released by incubating the monolayers in low pH buffer (2M NaCl, 20mM sodium acetate pH 4) for 5 min, followed by a wash with the same buffer (Nugent & Edelman 1992). At each time point non-specific binding of  $^{125}\text{I}$ -aFGF or  $^{125}\text{I}$ -bFGF was determined in the salt and acid washes of samples containing 50ug/ml of unlabeled FGF and these values subtracted from the experimental points.

In some experiments the effect of heparin on the rate of association of  $^{125}\text{I}$ -aFGF and  $^{125}\text{I}$ -bFGF with FGFR on heparinase-treated cells was also examined. Briefly, after preincubation of FGF with heparin, association was determined by incubating the cells with  $^{125}\text{I}$ -FGF in the presence and absence of heparin at 4°C for various periods of time up to 4 hours. At the end of each incubation period,  $^{125}\text{I}$ -FGF was removed by suction and the cells washed three times with 200ul/well of ice cold binding buffer, followed by salt and acid washes as described above. Nonspecific binding at each time point was determined by including an excess of unlabeled FGF (50ug/ml) in the assay mixtures and subtracting the  $^{125}\text{I}$ -FGF which remained bound, from the experimental points.



Additional experiments examined the effect of heparin and CR-heparin on the binding of  $^{125}\text{I}$ -FGFs to heparinase treated cells. Serial dilutions of either heparin or CR-heparin in binding buffer were added to an equal volume of either  $^{125}\text{I}$ -aFGF or  $^{125}\text{I}$ -bFGF (final concentration 20ng/ml) and incubated on ice with regular mixing for 30-60min. Nonspecific binding of  $^{125}\text{I}$ -aFGF and  $^{125}\text{I}$ -bFGF in the presence and absence of heparin or CR-heparin was determined by including an excess of unlabelled aFGF or bFGF (final concentration 50ug/ml) in the incubation mixtures. Heparin-FGF mixtures were added to heparinase treated BALB/c 3T3 cells (100ul/well), incubated for 4 hours on ice, and after washing once with ice cold medium,  $^{125}\text{I}$ -aFGF or  $^{125}\text{I}$ -bFGF bound to residual HSPG or FGFR released by exposing the cells to 2M NaCl and pH 4.0 solutions, respectively, as described above.

To determine the ability of chicken HRG (cHRG), human HRG (hHRG), ATIII, PF4, heparin, aFGF and bFGF to inhibit the binding of  $^{125}\text{I}$ -aFGF and  $^{125}\text{I}$ -bFGF to the 3T3 cell surface, cells were incubated with 95ul/well of each inhibitor at the concentration indicated for 1 hour on ice before adding 5ul of either  $^{125}\text{I}$ -aFGF or  $^{125}\text{I}$ -bFGF to each well to give a final concentration of 10ng/ml of  $^{125}\text{I}$ -FGF. The plate was incubated for a further 2 hours on ice before aspirating unbound  $^{125}\text{I}$ -FGF and washing cells three times with ice cold Hanks BSS/0.1% BSA. Cells were lysed by incubation with 100ul/well of PBS/0.5% Triton-X100 for 30min, mixed and transferred to counting tubes for counting by a gamma counter.

### **2.11. Binding of Radiolabeled aFGF, bFGF and cHRG to Extracellular Matrices**

Extracellular matrices (ECM) were prepared as follows: bovine corneal endothelial cells at passage 6-12 were seeded into 96-well plates (Nunc) at a concentration of  $1.25 \times 10^5$  cells/ml. Cells were grown to confluence and confluent monolayers denuded of cells by incubating monolayers with



150ul/well of 0.02M ammonium hydroxide for 5 min at room temperature, followed by a PBS rinse and incubation of each well with 150ul of 0.5% TX-100 in PBS for 30min at 37°C. Lysed cells were removed to expose the subendothelial matrix. ECMs were stored in PBS/ 0.1% azide at 4°C until required and washed three times with 200ul/well of PBS just prior to use. To determine the relative affinities of  $^{125}\text{I}$ -HRG,  $^{125}\text{I}$ -aFGF and  $^{125}\text{I}$ -bFGF for ECM HSPGs, serial dilutions of each radiolabeled molecule, (supplemented with unlabeled molecule) were made in Hanks BSS/0.1% BSA and 100ul of each dilution mixed with 100ul of medium or medium containing heparin (500ug/ml). Following incubation for 1 hour on ice, the mixtures (50ul/well) were transferred to ECM containing wells and incubated for 2 hours on ice. The unbound radiolabeled molecules were removed by aspiration and the wells washed three times with 200ul/well of ice-cold Hanks BSS/0.1% BSA. The ECMs and bound radiolabeled molecules were solubilized by incubating each well with 200ul of 4M guanidine HCl /2% Triton-X100, overnight at 4°C. The amount of radiolabeled aFGF, bFGF and cHRG associated with the ECM was then determined by a gamma counter.

In binding-inhibition experiments, 50ul of either cHRG, hHRG, PF4, ATIII, heparin, aFGF or bFGF were added per well to give the final concentration required. The inhibitors were allowed to bind to the ECM for 1 hour at 4°C before 5ul/well of either  $^{125}\text{I}$ -bFGF (22ng/ml) or  $^{125}\text{I}$ -aFGF (440ng/ml) was added to each well. Incubation was for 2 hours at 4°C before the reaction was terminated with the removal of unbound growth factor by washing and the release of radiolabeled growth factor bound to the ECM, as described above.

#### **2.12. Binding Affinity of Radiolabeled Heparin and CR-Heparin for Immobilized aFGF and bFGF**

Acidic and basic FGF were diluted from 200ug/ml to 5ug/ml in PBS, and round bottom polyvinylchloride microtitration plates (PVC)(Dynatech Laboratories, Inc.



VA, USA) were either coated with 50ul/well of aFGF or bFGF, at a constant concentration of 5ug/ml, and the plates left at 4°C overnight. Unbound FGF was removed from the plates by suction and the plates dunked twice in a PBS bath. Serial dilutions of radiolabeled heparin (0.003-6.5ug/ml) and CR-heparin (0.003-5.3ug/ml) were prepared in PBS/0.1% BSA and 50ul of each, added to quadruplicate wells. Control wells received a 100-fold excess of the appropriate unlabeled heparin in addition to each concentration of radiolabeled heparin. After a 2 hour incubation on ice, unbound radiolabelled heparins were aspirated and the wells washed four times with 200ul/well PBS. The wells were removed from the plate by a hot wire and counted by an Auto-gamma 5650 counter (Packard Instrument International). Specific binding of  $^{125}\text{I}$ -heparin to FGF was determined by subtracting the mean binding of  $^{125}\text{I}$ -heparin in the presence of excess unlabeled heparin from the total  $^{125}\text{I}$ -FGF binding. Scatchard analysis was performed with a computer program, "Scatchard Plot".

### **2.13. Rose Bengal Cell Adhesion Assay**

A modified version of the cell-adhesion assay developed by Ishihara et al., (1992) was used to investigate the interaction of BALB/c 3T3 cell surface HSPG with FGFs immobilized on plastic. Ninety-six well round bottom polyvinylchloride (PVC) Microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 50ul/well of either aFGF, bFGF, cHRG or hHRG (at the concentrations indicated) overnight at 4°C. Wells were aspirated, the plate dunked twice in a PBS bath, and non-specific binding sites blocked by incubation with Hanks BSS/0.1% BSA, pH 7.0 at 37°C for 1 hour. BALB/c 3T3 cells were suspended in Hanks BSS/0.1% BSA at a cell density of  $5 \times 10^6$  cells/ml, 0.1ml applied to each coated well and incubated at 37°C for 1 hour. The plate was flicked to remove unbound cells and 100ul/well of 0.25% Rose Bengal dye (Koch-Light Laboratories Ltd, Colnbrook Berks, England) in PBS for 3min at room temperature. Rose Bengal stains the nuclei and cytoplasm of both live and dead cells (O'Neill & Parish 1983). The unadsorbed dye was



removed by flicking the plate and dunking it twice in two separate PBS baths. The plate was allowed to drain before adding 200ul/well of 50% ethanol in PBS. Each well received a constant amount of mixing with a multichannel pipette to allow liberation of the dye from the cells. Nonspecific binding of the dye to FGF-coated and uncoated wells in the absence of cells was also determined and subtracted from experimental points. The relative number of cells in each well was quantified by determination of each well's optical density ( $\lambda_1=540\text{nm}$ ,  $\lambda_2=650\text{nm}$ ) using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA).

In order to determine the optimum time and temperature for incubation of cells on FGF-coated PVC wells, 100ul/well of cells ( $5 \times 10^6$  cells/ml) were added in triplicate to aFGF (5ug/ml)-coated wells and incubated at either room temperature or  $37^\circ\text{C}$  for the times indicated. Control wells were not coated with FGF but received cells at the same times as experimental wells.

To determine the optimal cell concentration to use in this assay, doubling dilutions of cells in Hanks BSS/0.1% BSA were prepared, ranging between  $1 \times 10^6$  cells/well and  $3.9 \times 10^3$  cells/well and incubated with HRG-coated (10ug/ml) wells for 1 hour at  $37^\circ\text{C}$ . To determine the optimal concentrations of aFGF, bFGF, cHRG and hHRG with which to coat the wells, wells of a plate were coated with a range of concentrations of either aFGF, bFGF, cHRG or hHRG in triplicate. 100ul/well of cells at a final concentration of  $2.5 \times 10^6$  cells/ml was added to wells coated with FGF and 100ul/well of cells at a final concentration of  $5 \times 10^6$  cell/ml added to HRG coated wells. The assay was then performed as described above. To determine whether acidic and basic FGF adhere to the PVC plate to an equal extent, quadruplicate wells were coated with either 50ul/well of  $^{125}\text{I}$ -bFGF or  $^{125}\text{I}$ -aFGF, both at 313ng/ml, and left overnight at  $4^\circ\text{C}$ . Unbound FGF was removed by washing twice, the two washes being collected. 100ul/well of Hanks BSS/0.1% BSA was then added, the plate incubated for 1 hour at  $37^\circ\text{C}$ , supernatants collected and individual



wells detached from the plate by a hot wire. The amount of labeled FGF in each wash and that which remained bound to the wells was determined by a gamma counter.

The ability of various GAGs (100ug/ml) to inhibit the binding of cell-surface HSPGs to aFGF, bFGF, cHRG and hHRG was also tested. Fifty ul/well of GAG was incubated with FGF- or HRG-coated wells for 1 hour at 4°C before 50ul of cells ( $5 \times 10^6$  cells/ml) were added to each well and incubated at 37°C for 1 hour. To titrate the inhibitory activity of some GAGs or proteins doubling dilutions of each inhibitor were prepared in Hanks BSS/0.1% BSA pH 7.0 and 50ul/well of each concentration aliquoted in triplicate into FGF or HRG coated wells and the same procedure followed as described above. Control wells received medium without inhibitor.

To determine whether PF4, ATIII, cHRG, hHRG, aFGF and bFGF cross react with the same HSPGs on the 3T3 cell surface, each of these factors were tested for their ability to inhibit the binding of 3T3 cells to PVC plates coated with one of the following: aFGF, bFGF, cHRG or hHRG. Serial dilutions of each of PF4, cHRG, hHRG, aFGF and bFGF were prepared and equal volumes of each dilution incubated with an equal volume of cells ( $5 \times 10^6$  cells/ml) for 2 hours at 4°C before aliquotting 100ul of each sample to triplicate wells coated with FGF or HRG. Control wells received cells which had been preincubated with medium in place of growth factor.

#### **2.14. Fluorescence Flow Cytometry**

In order to confirm the results of the Rose Bengal plate assays, an assay was developed which utilized the ability of a FACScan to quantify the binding of a fluoresceinated form of cHRG to 3T3 cells. FITC-conjugation of chicken HRG was performed by Dr C. Parish as follows: 1ml cHRG (2.14mg/ml) in PBS was dialyzed overnight at 4°C against 500ml of 0.05M boric acid, 0.2M NaCl,



pH9.2. 10mg of fluorescein-5-isothiocyanate (FITC; Molecular Probes, Eugene, OR) were dissolved in 2.0ml DMSO and 50ul of FITC-DMSO immediately added to the cHRG solution. Incubation was for 2 hours at room temperature in the dark. The sample was applied to a PD-10 column and fluorescent HRG eluted in PBS to give a final concentration of 1mg/ml.

To test the ability of cHRG, hHRG, aFGF, bFGF and PF4 to inhibit the binding of FITC-cHRG to 3T3 cells, cells were grown to confluence in DMEM/20% FCS/1% L-glutamine/20mM Hepes, detached with PBS/EDTA and adjusted to  $5 \times 10^6$  cells/ml in Hanks BSS/0.1% BSA pH7.0. To wells of a 96-well V-bottom plate (Serocluster Costar; Cambridge, Mass.) were added 20ul of cells and 20ul of inhibitor, spanning the concentration ranges required. The plate was mixed gently and incubated on ice for 30min. Two microlitres of FITC-cHRG (1mg/ml) were added to each well and after further mixing, the plate incubated on ice for 1 hour. Unbound FITC-cHRG was removed by washing the wells 3 times with 150ul/well of ice cold Hanks BSS/0.1% BSA pH7.0, the cells being pelleted by a GS-6R centrifuge (Beckman, Sydney, Australia) (at 200g force for 1min at 4°C). Cell pellets were resuspended in 100ul of ice cold Hanks BSS/0.1% BSA, and the fluorescence intensity of each sample determined by a FACScan (Becton Dickinson, Mountain View, CA). Control samples contained medium in place of inhibitor, or medium in place of FITC-cHRG.

To test the ability of heparin to inhibit the binding of FITC-cHRG to 3T3 cells, serial dilutions of 20ul FITC-cHRG were made in a V-bottom plate and 2ul heparin (2mg/ml) added to each well. After incubating the plate on ice for 30min, 20ul of 3T3 cells ( $5 \times 10^6$  cell/ml) were added to each well. Control samples included HRG in the absence of heparin, and cells in the presence and absence of heparin without HRG. The plate was mixed gently and incubated for a further 30min on ice. Unbound FITC-cHRG were removed by



washing the plate three times in the same manner as described above and fluorescence quantified by a FACScan.

In some experiments, a rabbit anti-CHRG antiserum was also used to quantify the binding of CHRG to HSPGs on the 3T3 cell surface. To determine whether the medium used affects the ability of CHRG to bind to cells, the assay was performed in DMEM/1% L-glutamine/20mM Hepes; F15 (Minimum Essential Medium 410-1500, Gibco) /1% L-glutamine/20mM Hepes, RPMI 1640 (Gibco) /1% L-glutamine/20mM Hepes; and Medium199 (Gibco) /1% L-glutamine/20mM Hepes. Cells were prepared in PBS/EDTA, transferred to V-bottom wells of a 96-well plate, pelleted, the supernatant discarded and cells resuspended in 20ul/well of CHRG diluted to 100ug/ml in one of the media mentioned above. Incubation was for 2 hours on ice. Unbound HRG was removed by washing samples three times in the appropriate medium. Rabbit anti-CHRG antiserum (prepared and provided by Dr C. Parish) was then added (20ul/well) and the plate incubated for 30min on ice. Unbound antibody was removed by washing the plate three times as described above and 20ul/well of Protein A-FITC (Boehringer-Mannheim, Mannheim, Germany) added. Incubation was for a further 30min on ice before unbound FITC-Protein A was removed by washing 3 times and cell pellets resuspended in 100ul/well of the appropriate medium. Samples were then analyzed by a FACScan.

### **2.15. Soluble FGF Receptor Binding Assay**

Comparison of the abilities of heparin and CR-heparin to promote the binding of  $^{125}\text{I}$ -aFGF and  $^{125}\text{I}$ -bFGF to an FGFR in a cell-free system were tested by Dr David Ornitz (Washington University, St. Louis, Missouri) using a soluble form of the murine FGF receptor 1 (mFR1). A plasmid containing mFR1 fused to human placental alkaline phosphatase (AP) cDNA (FR1AP) was introduced into NIH 3T3 cells, and monoclonal antibodies to placental AP coupled to Sepharose used to immunoabsorb FR1AP from the conditioned medium. The



components of the soluble binding reaction mixture included FR1AP-conditioned medium (0.24 OD units/min),  $^{125}\text{I}$ -bFGF or  $^{125}\text{I}$ -aFGF, heparin or CR-heparin, 20ul of a 2 x slurry of anti-AP monoclonal antibodies coupled to Sepharose, DMEM and 0.1% BSA in a volume of 250ul. The components were mixed at room temperature and rotated for 2-3 hours at 4°C. Bound receptor or ligand was recovered by centrifuging (10s at 6000rpm at 4°C in a microcentrifuge) and washing two times with 500ul of ice-cold PBS.  $^{125}\text{I}$ -FGF binding was determined by counting tubes directly in a  $\gamma$  counter (Ornitz & Leder 1992).

### **2.16. Effect of cHRG on Cell Attachment**

Bacteriological petri dishes (Johns Scientific, Sydney, Australia) were incubated overnight at 4°C with 20ml of either PBS or PBS containing 10ug/ml of HRG. PBS and unbound cHRG were aspirated and the dishes washed three times with 10ml Hanks BSS/0.1% BSA and non-specific binding sites blocked by incubation with 10ml Hanks BSS/0.1% BSA pH 7.0 at 37°C for 1 hour. After removal of the blocking buffer, each petri dish received 10ml of a BALB/c 3T3 cell suspension ( $0.6 \times 10^5$  cells/ml), and the cells were left to adhere to the dishes for 2 hours at 37°C before being examined for spreading under a light microscope.

### **2.17. Mitogenic assays**

The mitogenic activity of aFGF and bFGF was determined by measuring their ability to stimulate DNA synthesis in HUVE cells and in serum starved BALB/c 3T3 cells, as determined by [methyl- $^3\text{H}$ ]thymidine incorporation. To compare the abilities of heparin and CR-heparin to potentiate aFGF activity over time, equal volumes of aFGF and medium, aFGF and heparin, and aFGF and CR-heparin, were incubated together for 1 hour on ice with regular mixing, before being added to serum starved 3T3 cells (100ul/well) to give final concentrations of 10ng/ml for aFGF and 50ug/ml for each heparin. [Methyl- $^3\text{H}$ ]thymidine



(0.5uCi/well) (60Ci/mmol) (Amersham) was added for the final 24 hours of each treatment. Incubation was stopped by freezing and thawing the cultures three times, the assay harvested with a 1295-004 Betaplate 96-well harvester (Pharmacia, Uppsala, Sweden) and the radioactivity counted with a liquid scintillation counter (Pharmacia).

To compare the effects of heparin on the dose response curves of acidic and basic FGF for BALB/c 3T3 cells and HUVE cells, serial dilutions of acidic and basic FGF in the culture medium were prepared and equal volumes of each FGF dilution mixed with either medium or heparin to give a final heparin concentration of 5ug/ml. Samples were mixed and incubated at 4°C for 30min. A constant volume of HUVE cells ( $2.5 \times 10^4$  cells/well) was added to each sample tube and 100ul of sample added to quadruplicate wells of a 96-well plate (Nunc) precoated with 0.1% gelatin. Control wells received cells and medium without FGF or heparin. In the case of BALB/c 3T3 cells, after incubation of the different FGF dilutions with and without heparin, 100ul of each dilution was added to quadruplicate wells of 3T3 cell monolayers. Control wells received medium without FGF or heparin. Incubation was for 24 hours before the addition of [methyl- $^3\text{H}$ ]thymidine for a further 24 hours.

In order to determine whether high concentrations of heparin and CR-heparin would inhibit aFGF-induced mitogenesis in cells lacking HSPGs, cells were cultured in chlorate and sulfate-free medium (BME) for 48 hours, as described by Rapraeger et al.,(1991). The cells were treated with heparinase 1 (2 units/well) to remove residual cell surface HSPGs before receiving treatments. Control cultures received BME in place of heparinase and were subjected to the same washing procedure. Treatments consisted of aFGF (20ng/ml) in the presence and absence of 0.1, 10, and 1000ug/ml of heparin and CR-heparin. Incubation mixtures for heparinase-treated cells were prepared in BME and 30mM chlorate while cells not treated with heparinase received the same



incubation mixtures prepared in BME and 0.8mM sulfate. Incubation was for 24 hours at 37°C before [methyl-<sup>3</sup>H]thymidine was added for a further 24 hours.



## Chapter 3

### Acidic and Basic FGF Bind with Differing Affinity to the Same HSPG on BALB/c 3T3 Cells: Implications for Potentiation of Growth Factor Action by Heparin

#### 3.1. Introduction

Acidic and basic FGF bind to two classes of binding site on the cell surface, the FGFR which is regarded as a "high affinity" binding site on the basis of low  $K_d$  values ( $K_d=10^{-9}$ - $10^{-12}$ M) (Neufeld & Gospodarowicz 1985; Moenner et al 1986; Olwin & Hauschka 1986) and HSPGs regarded as "low affinity" binding sites on the basis of relatively high  $K_d$  values ( $K_d=10^{-8}$ - $10^{-9}$ M) (Moscatelli 1987). HSPGs on the cell surface generally outnumber FGFRs by 1-3 orders of magnitude (Moscatelli 1987; Burgess & Maciag 1989). Furthermore, it is generally accepted that the presence of either HSPG or heparin is required for the binding of FGFs to the "high affinity" receptor. This study aimed to clarify several aspects of the interaction of FGFs with HSPGs.

First, I have examined whether both acidic and basic FGF recognize similar or different motifs within the HS chains of cell surface HSPGs and have determined their relative binding affinities for these HS motifs.

Second, this study examined the importance of HSPG in stabilizing the interaction of FGFs with their "high affinity" receptor. Two models have been proposed to explain how HSPGs promote the binding of FGFs to their receptors. In one model it is proposed that HSPGs induce a conformational change in FGF which enables the growth factor to bind with high affinity to its receptor (Yayon et al., 1991). In a second model, Nugent & Edelman (1992) have proposed that formation of a "ternary complex" is required which does not involve a conformational change in the molecule, but requires the simultaneous binding of FGF to FGFR and HSPG for stable binding to occur. I have undertaken a comparative study, using both aFGF and bFGF, to determine the



relative importance of HSPG in stabilizing the FGF/FGFR complex. In particular, the role of the "ternary complex" model of FGF binding was examined for both growth factors.

Finally, based on these observations an attempt has been made to explain why heparin potentiates aFGF action but has little or no potentiating effect on bFGF function. Of particular interest was whether differences in the affinity of the two FGFs for cell surface HSPGs can explain why acidic but not basic FGF is potentiated by heparin.

### **3.2. Results**

#### **3.2.1. Binding of Acidic and Basic FGF to Cell Surface HSPGs**

In order to examine the interaction of acidic and basic FGFs with BALB/c 3T3 cell surface HSPGs, an assay based on that of Ishihara et al.,(1992) was developed to determine whether acidic and basic FGFs interact with the same HSPGs, and if so, whether they differ in their affinity for these HSPGs. The assay utilized the ability of the FGFs to adhere to 96-well PVC plates and of cells to bind to the FGF-coated wells via their cell surface HSPGs. Bound cells were then quantified using the vital dye, Rose Bengal.

Initially, the optimum time and temperature for incubation of the cells with the FGF coated plate was determined (Fig. 3.1). Binding of cells to plates coated with aFGF was shown to occur most rapidly at 37°C, a little slower at room temperature and not at all at 4°C. This temperature effect is probably due to a rapid redistribution of ligands on the cell surface at 37°C which would allow more HSPGs to come into contact with the factor on the plate, and thereby result in multivalent binding. Subsequent assays were incubated for 1 hour at 37°C with the optimum cell number of  $2.5 \times 10^5$  cells/well.



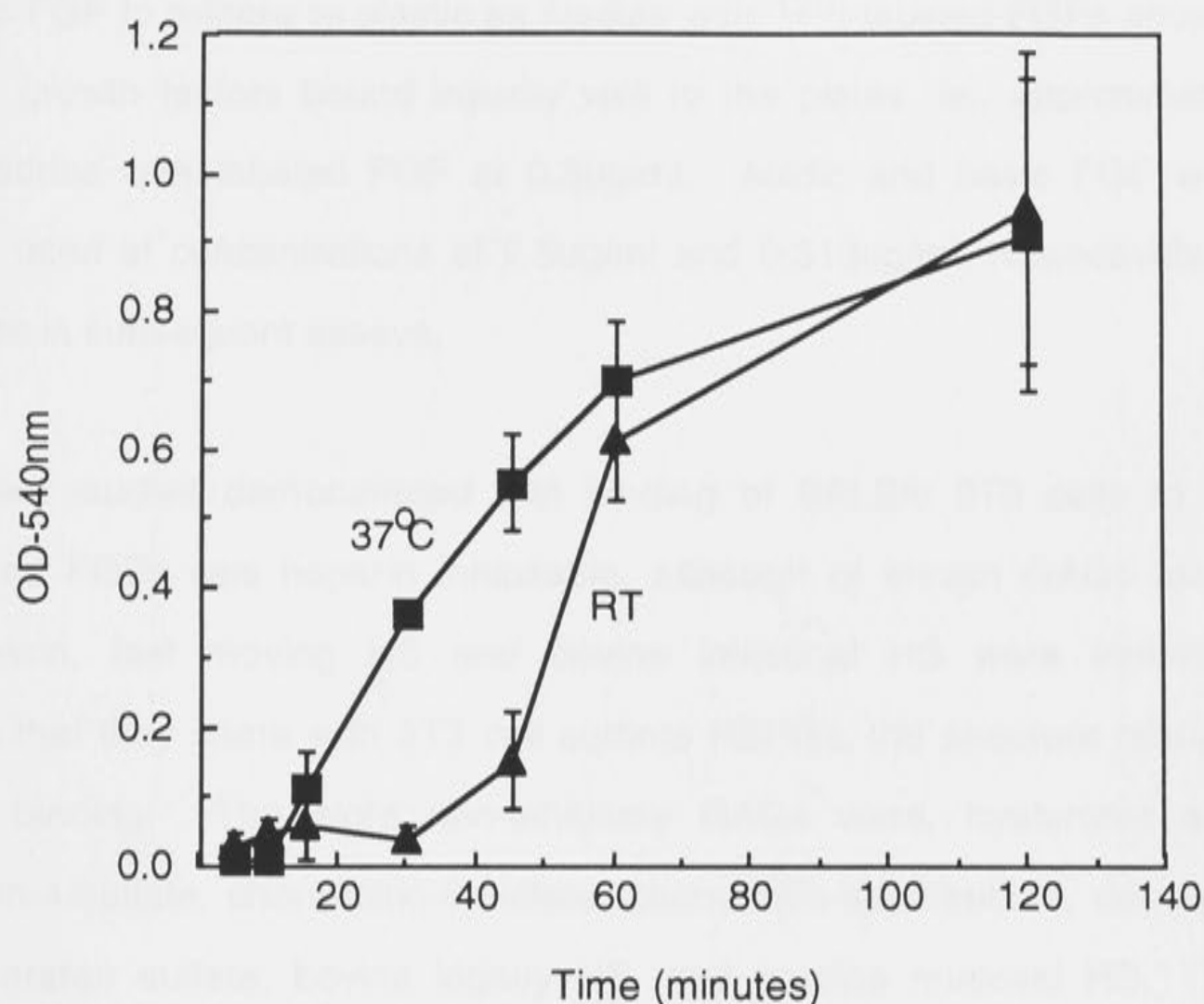


FIGURE 3.1: Determination of the optimum time and temperature for binding of BALB/c 3T3 cells ( $5 \times 10^5$  cells/well) to PVC wells coated with aFGF (5  $\mu$ g/ml). Incubation was at 37°C (■) and at room temperature (▲) for the times indicated. There was no cell binding at 4°C (data not shown). Values represent means  $\pm$  SEM ( $n=3$ ), the number of cells remaining bound to each well being quantified by Rose Bengal staining.



The optimal FGF concentration to be used for coating the wells was also determined (Fig. 3.2). It was found that for optimum cell binding, an approximately 8 fold higher concentration of aFGF, than bFGF, was required for coating. This difference was not due to differences in the ability of acidic and basic FGF to adhere to plastic as studies with  $^{125}\text{I}$ -labeled FGFs showed that both growth factors bound equally well to the plates, i.e., approximately 15% of added radiolabeled FGF at 0.3ug/ml. Acidic and basic FGF were generally used at concentrations of 2.5ug/ml and 0.313ug/ml, respectively, to coat plates in subsequent assays.

Subsequent studies demonstrated that binding of BALB/c 3T3 cells to the immobilized FGFs was heparin inhibitable, although of eleven GAGs tested only heparin, fast moving HS and bovine intestinal HS were inhibitory, indicating that they share with 3T3 cell surface HSPGs, the structure required for FGF binding. The eight non-inhibitory GAGs were, hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, chondroitin-4,6-disulfate, dermatan sulfate, keratan sulfate, bovine kidney HS, and porcine mucosal HS. The complete inhibition curves for heparin, fast moving HS and bovine intestinal HS are depicted in Figure 3.3. for bFGF, and Figure 3.4. for aFGF. For both growth factors, heparin was the most potent inhibitor. Fast moving HS was, on a weight basis, 480 fold less effective at inhibiting aFGF and 600 fold less effective at inhibiting bFGF binding than heparin. Bovine intestinal HS, at high concentrations, also inhibited bFGF binding but this inhibition was not observed with aFGF. This difference suggests that there may be some subtle differences in the HS motifs recognized by acidic and basic FGF.

Figure 3.5. shows that both soluble aFGF and bFGF can totally block the binding of immobilized bFGF to HSPGs on BALB/c 3T3 cells. Similarly, both FGFs totally inhibited immobilized aFGF from binding to 3T3 cells (Fig. 3.6). Thus, acidic and basic FGF cross react with the same species of cell surface



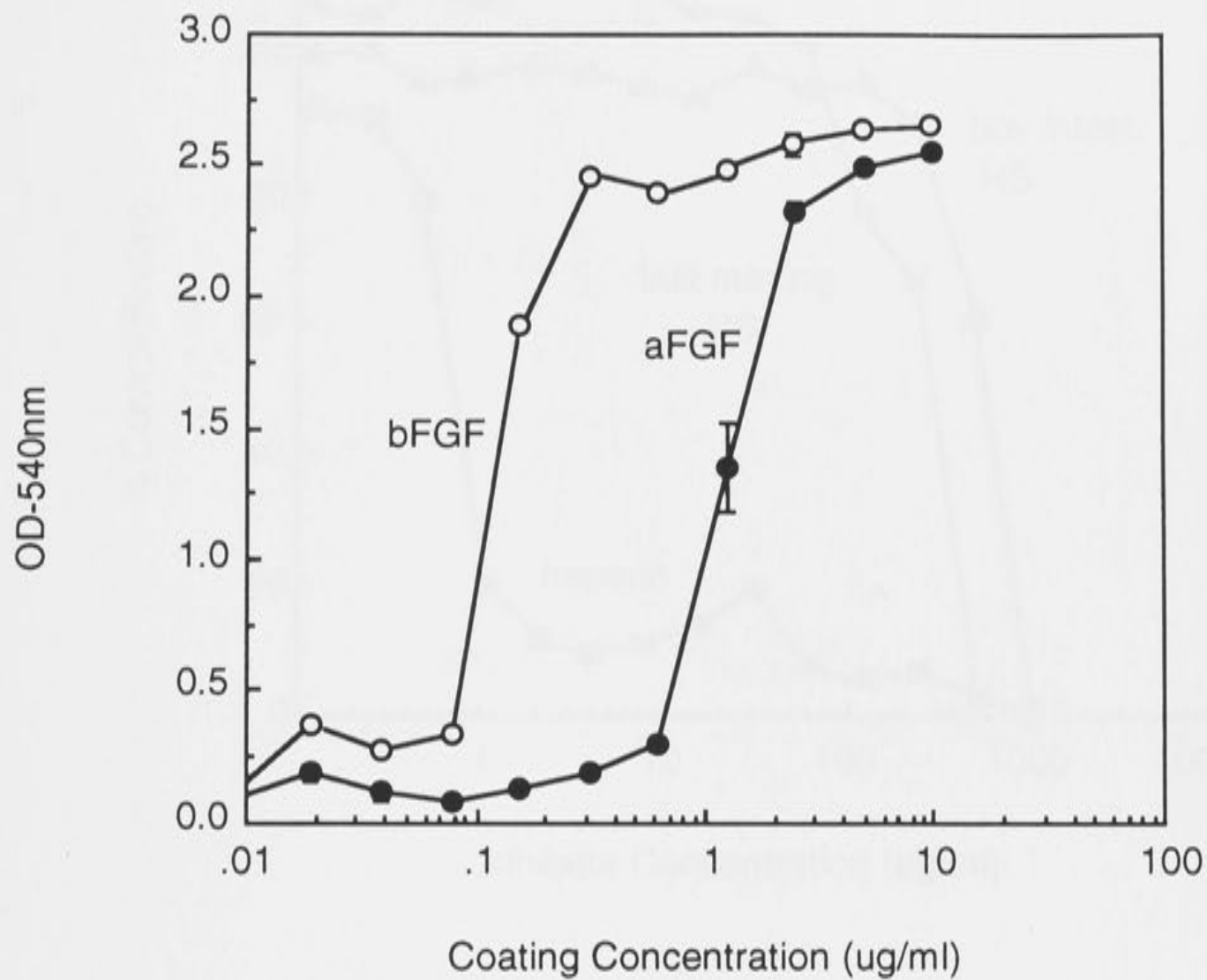


FIGURE 3.2: Determination of optimum coating concentration of FGF required for the binding of BALB/c 3T3 cells ( $2.5 \times 10^5$  cells/well) to PVC plates. Cell binding was performed for 60min at 37°C and the number of cells bound/well quantified by Rose Bengal staining. Values represent means  $\pm$  SEM (n=3) of one representative experiment.



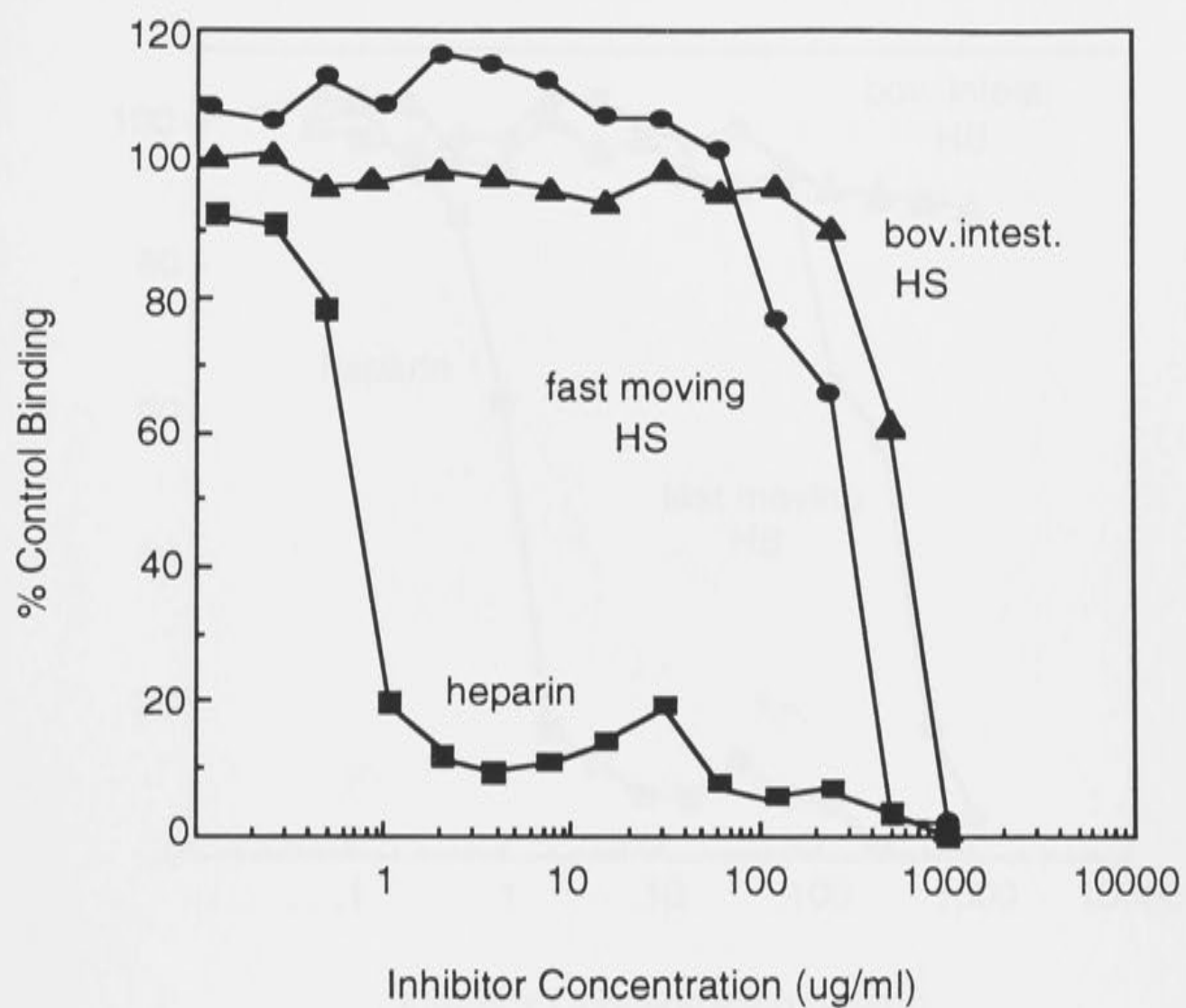


FIGURE 3.3: Comparison of the ability of heparin (■), fast moving heparan sulfate (●) and bovine intestinal heparan sulfate (▲) to inhibit the binding of BALB/c 3T3 cells to PVC wells coated with bFGF (0.625ug/ml). Heparin and heparan sulfates were incubated with plate-bound bFGF for 1 hour on ice before adding cells ( $2.5 \times 10^5$  cells/well) for 1 hour at 37°C. 100% binding represents that occurring in the absence of inhibitor. Each value represents the mean of triplicate treatments. Standard errors of the means were < 10%.



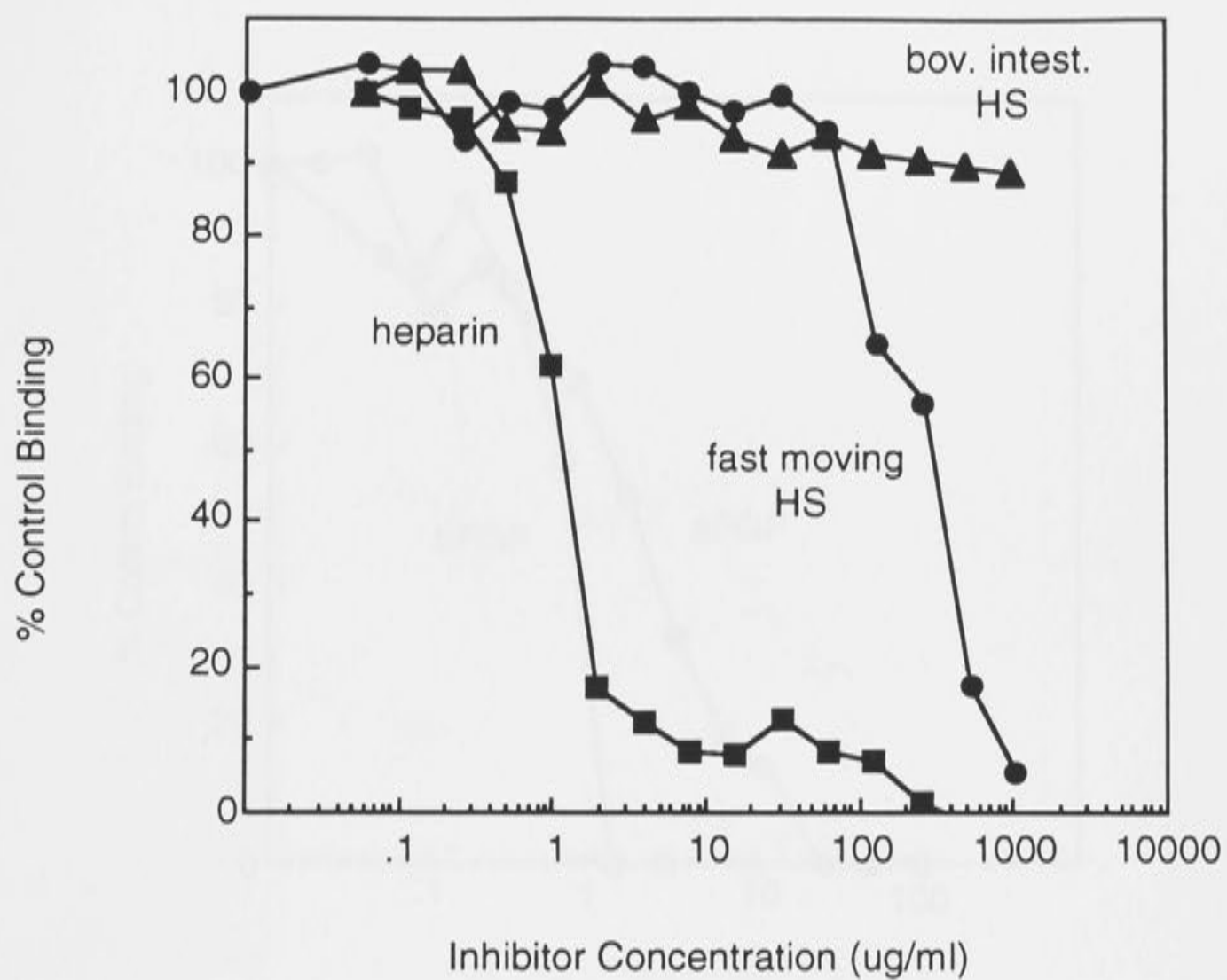


FIGURE 3.4: Comparison of the ability of heparin (■), fast moving heparan sulfate (●) and bovine intestinal heparan sulfate (▲) to inhibit the binding of BALB/c 3T3 cells to PVC wells coated with aFGF (2.5ug/ml). Assay conditions were as described in the legend of Figure 3.3.



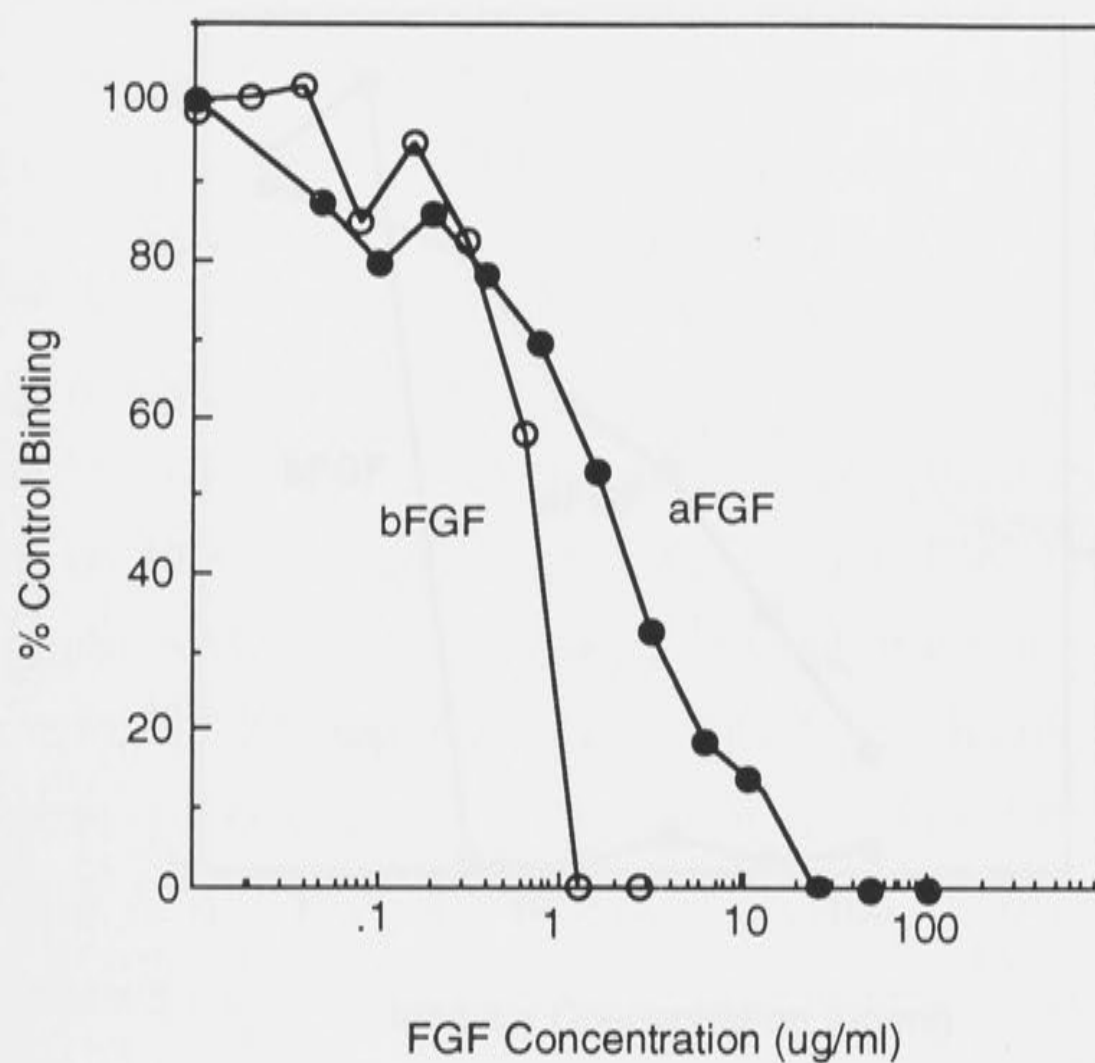


FIGURE 3.5: Ability of soluble aFGF and bFGF to inhibit the binding of BALB/c 3T3 cells to immobilized bFGF. Cells were incubated with a range of FGF concentrations for 2 hours on ice before being added to bFGF-coated wells for a further 1 hour incubation at 37°C. Each value represents the mean of triplicate treatments. 100% binding is the number of cells bound to bFGF in the absence of FGF inhibitor. Standard errors of means were < 5%.

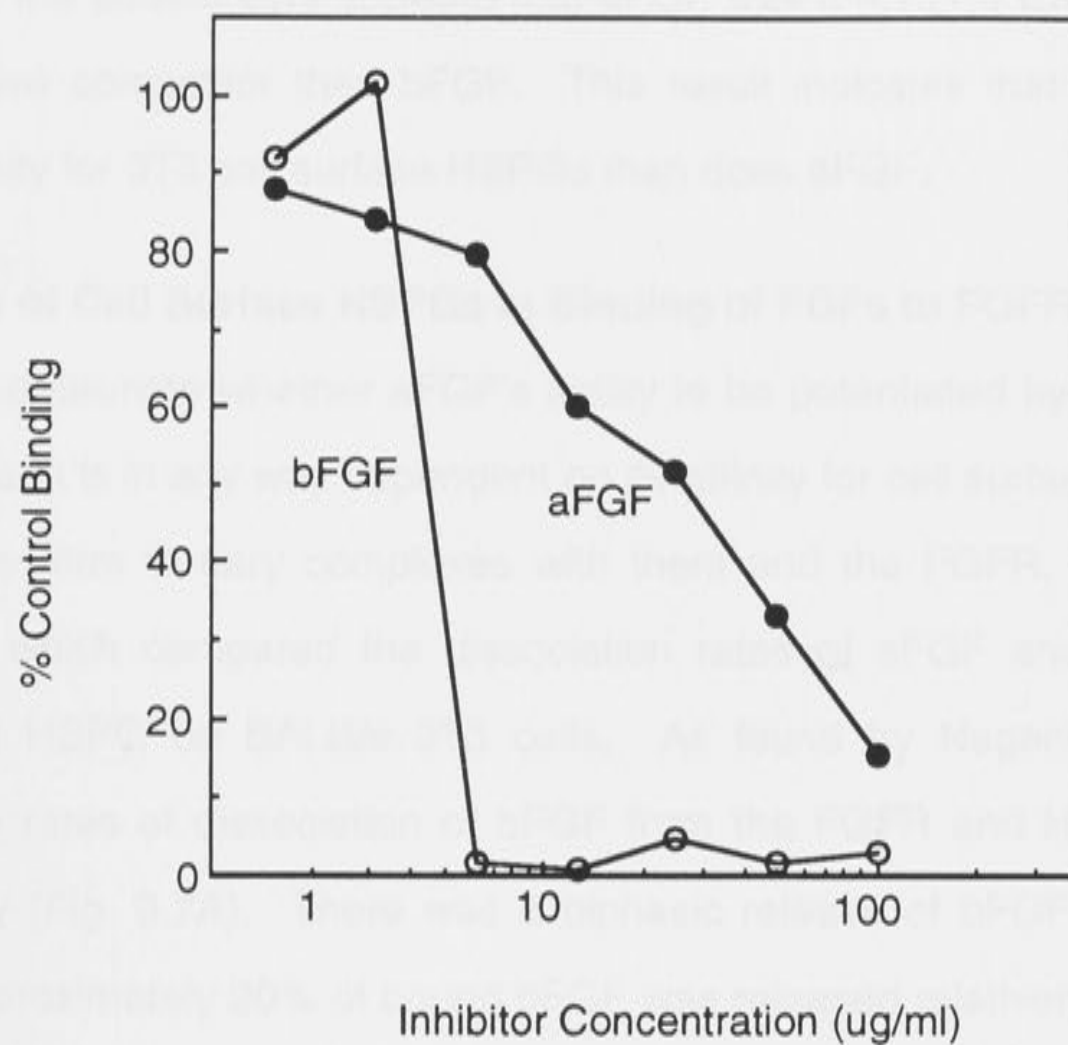


FIGURE 3.6: Ability of soluble aFGF and bFGF to inhibit the binding of BALB/c 3T3 cells to immobilized aFGF. Assay conditions and data presentation as described in legend of Figure 3.5.



HSPG on 3T3 cells. However, in both assays aFGF was a less effective inhibitor, the difference being 2.5 fold with immobilized bFGF (Fig. 3.5) and 7.8 fold with immobilized aFGF (Fig. 3.6) in the experiments shown. Subsequent replicate inhibition assays gave similar results irrespective of the immobilized FGF used, the pooled data showing that aFGF was a  $4.7 \pm 1.0$  (SEM,  $n=4$ ) fold less effective competitor than bFGF. This result indicates that bFGF has a higher affinity for 3T3 cell surface HSPGs than does aFGF.

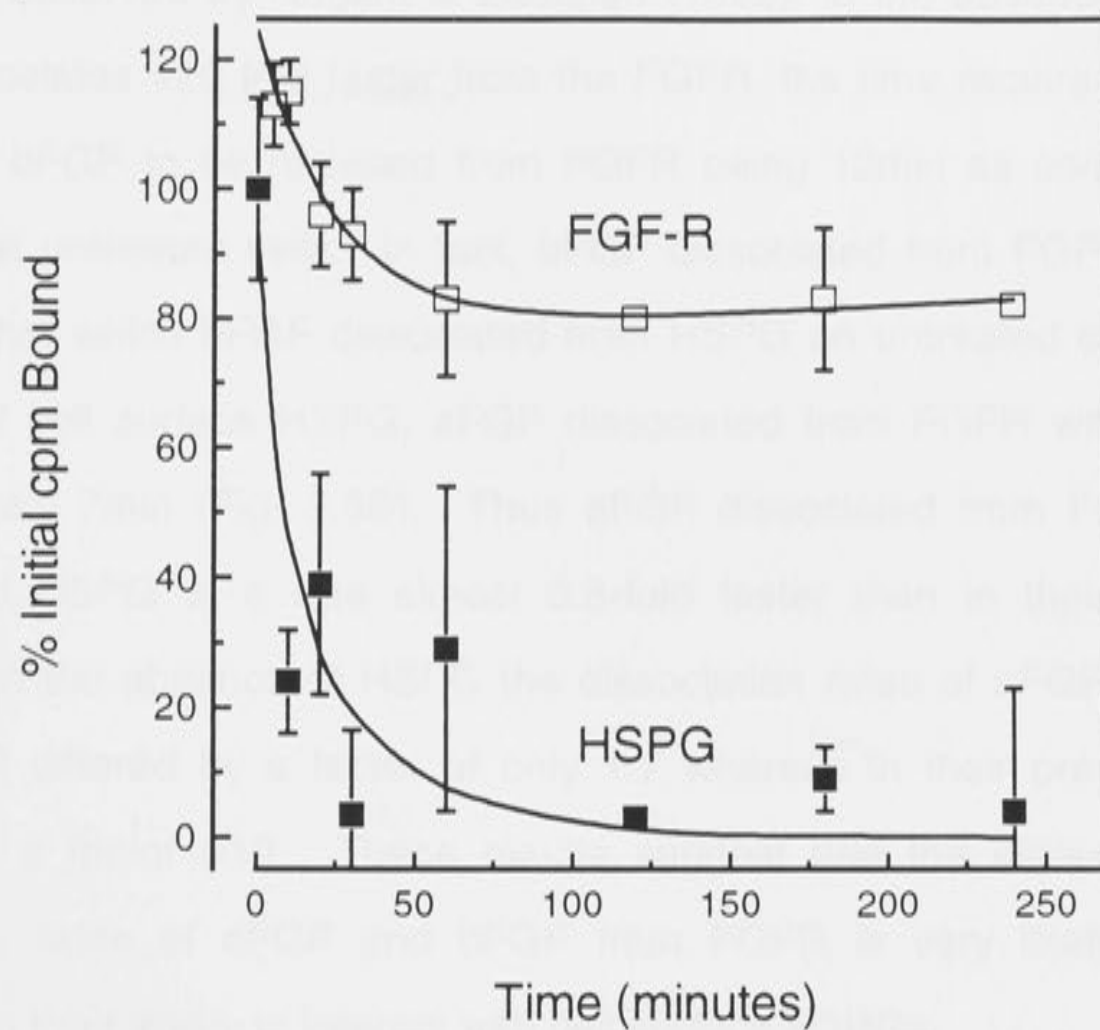
### **3.2.2. Role of Cell Surface HSPGs in Binding of FGFs to FGFR**

In order to determine whether aFGF's ability to be potentiated by exogenously added heparin is in any way dependent on its affinity for cell surface HSPG and its ability to form ternary complexes with them and the FGFR, studies were performed which compared the dissociation rates of aFGF and bFGF from FGFR and HSPG on BALB/c 3T3 cells. As found by Nugent & Edelman (1992), the rates of dissociation of bFGF from the FGFR and HSPG differed significantly (Fig. 3.7A). There was a biphasic release of bFGF from FGFR. Initially, approximately 20% of bound bFGF was released relatively rapidly from FGFR. This was followed by a second phase of dissociation during which the majority (80%) of FGFR-bound-bFGF dissociated extremely slowly, with the time required for half of the bound bFGF to be released ( $t_{1/2}$ ) from the FGFR being >250 min compared with approximately 9 min for the HSPG. In contrast, aFGF dissociated from the FGFR and HSPGs at similar rates,  $t_{1/2}$  being determined as 25 min and 16 min, respectively (Fig. 3.7B). Thus, aFGF dissociates from the FGFR considerably faster than does bFGF and this difference is reflected in their  $t_{1/2}$  values, being 25 min for aFGF and >250 min for bFGF dissociation from the FGFR. In contrast, the two growth factors dissociated from HSPG relatively rapidly and at similar rates, the  $t_{1/2}$  values for aFGF and bFGF being 16min and 9min, respectively.

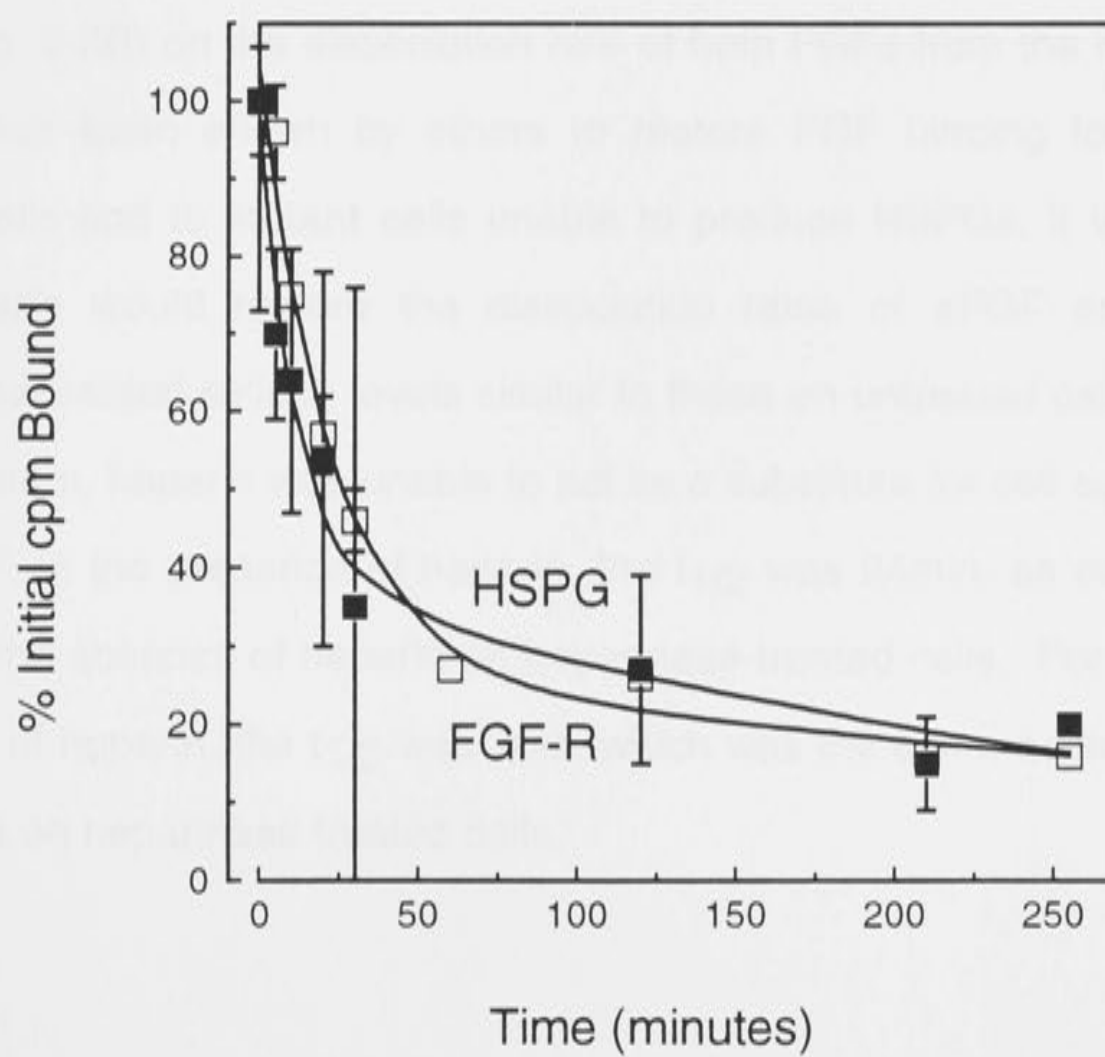
FIGURE 3.7: Dissociation of bFGF (A) and aFGF (B) from FGF-R and HSPG on intact BALB/c 3T3 cells. Dissociation was performed in the presence of an excess of unlabeled bFGF (7ug/ml) or aFGF (1ug/ml) to minimize rebinding of released  $^{125}\text{I}$ -FGF. Each data point represents the mean  $\pm$  SEM of triplicate treatments. Initial binding (100%) to FGF-R was  $893 \pm 38$  cpm and  $4701 \pm 166$  cpm for bFGF and aFGF, respectively. Initial binding (100%) to HSPG was  $434 \pm 60$  cpm and  $994 \pm 120$  cpm for bFGF (2ng/ml) and aFGF (10ng/ml), respectively.



A)



B)



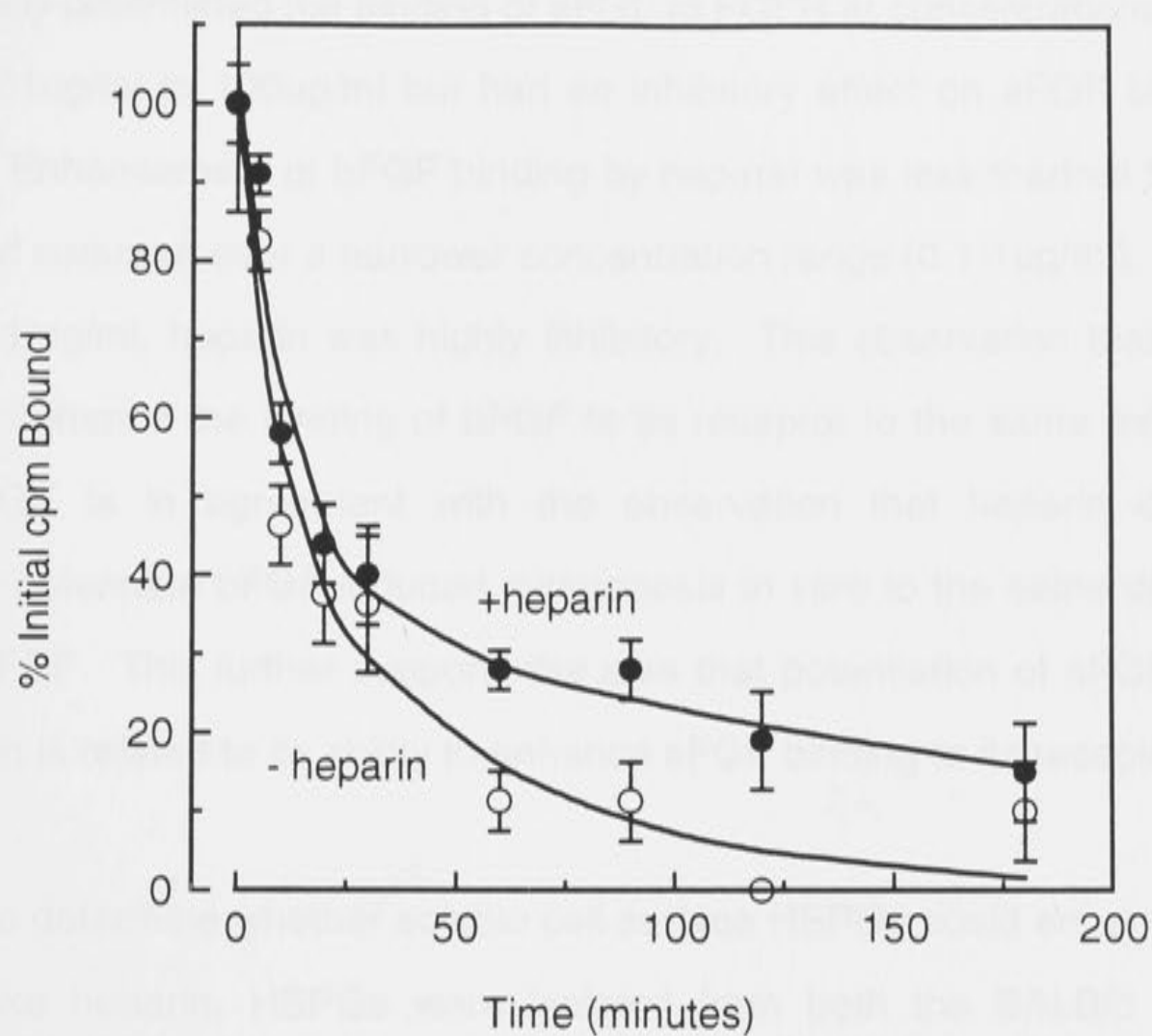
In order to determine whether heparin can act as a substitute for cell-surface HSPGs, cells were treated with heparinase to remove cell surface HSPG and the effect of heparin on the dissociation of bFGF from the FGFR observed (Fig. 3.8A). As observed by Nugent & Edelman (1992), in the absence of HSPG, bFGF dissociates >20 fold faster from the FGFR, the time required for half of the bound bFGF to be released from FGFR being 12min as compared with >250min on untreated cells. In fact, bFGF dissociated from FGFR at a rate similar to that which bFGF dissociated from HSPG on untreated cells. In the absence of cell surface HSPG, aFGF dissociated from FGFR with a  $t_{1/2}$  of approximately 7min (Fig. 3.8B). Thus aFGF dissociated from FGFR in the absence of HSPG at a rate almost 3.5-fold faster than in their presence. Therefore in the absence of HSPG the dissociation rates of aFGF and bFGF from FGFR differed by a factor of only 1.7 whereas in their presence, they differed by a factor >10. These results suggest that the difference in the dissociation rates of aFGF and bFGF from FGFR is very likely due to a difference in their ability to interact with cell surface HSPGs.

The presence of heparin during FGF binding had very little (Fig. 3.8A) or no effect (Fig. 3.8B) on the dissociation rate of both FGFs from the FGFR. Since heparin has been shown by others to restore FGF binding to heparinase-treated cells and to mutant cells unable to produce HSPGs, it was expected that heparin would restore the dissociation rates of aFGF and bFGF on heparinase-treated cells to levels similar to those on untreated cells. However, in this system, heparin was unable to act as a substitute for cell surface HSPG. For bFGF, in the presence of heparin, the  $t_{1/2}$  was 24min, as compared with 12min in the absence of heparin on heparinase-treated cells. For aFGF, in the presence of heparin, the  $t_{1/2}$  was 7min which was the same as in the absence of heparin on heparinase treated cells.

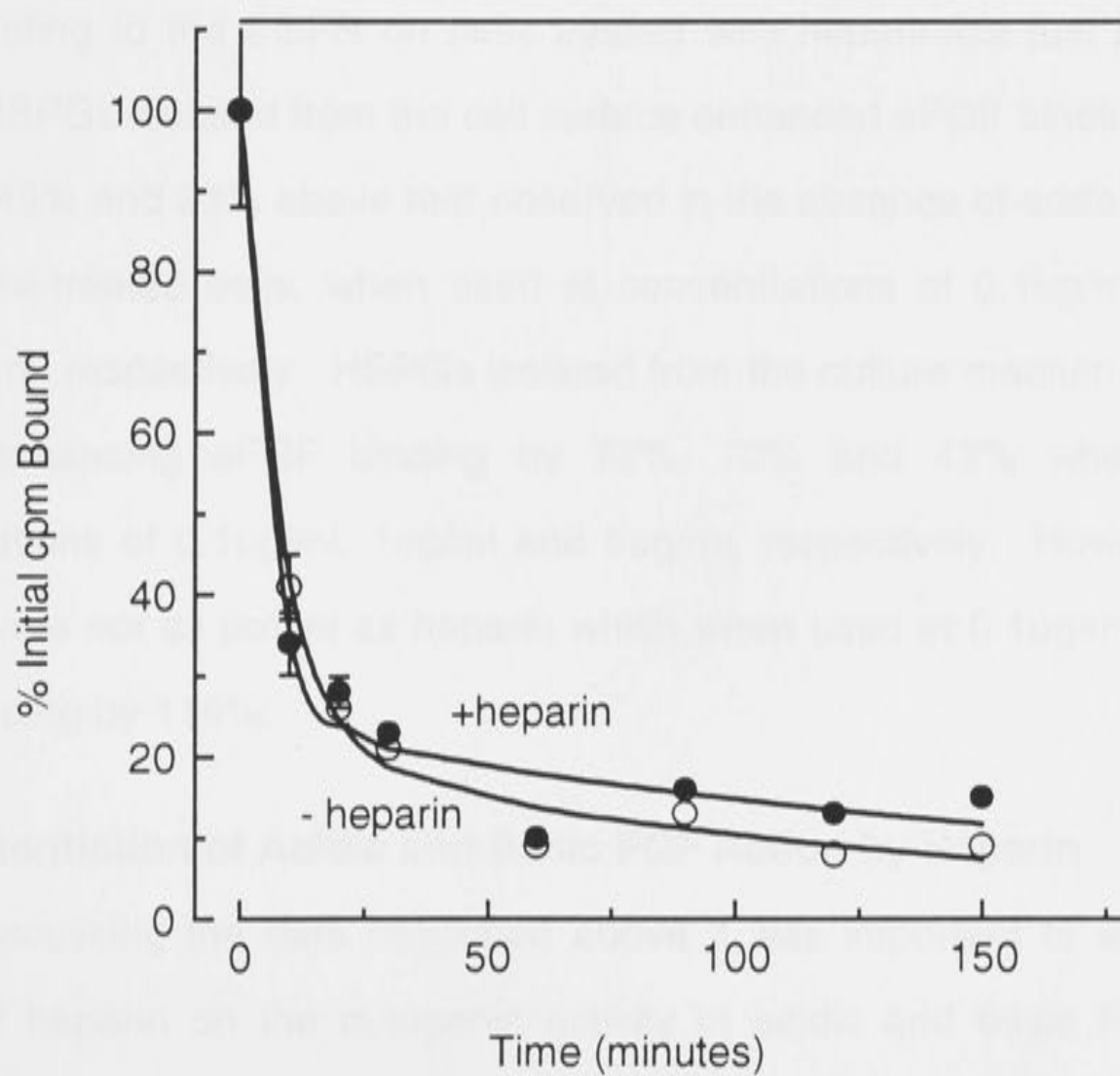


FIGURE 3.8: Dissociation of bFGF (A) and aFGF (B) from FGF-R on heparinase-treated BALB/c 3T3 cells in the presence and absence of heparin (0.1ug/ml). Dissociation was performed in the presence of an excess of unlabeled bFGF (7ug/ml) or aFGF (1ug/ml) to minimize rebinding of released  $^{125}\text{I}$ -FGF. Each data point represents the mean  $\pm$  SEM of triplicate treatments. Initial binding (100%) to FGF-R in the absence of heparin was  $1170 \pm 129$  cpm and  $1120 \pm 14$  cpm for bFGF and aFGF, respectively. Initial binding (100%) to FGF-R in the presence of heparin was  $1092 \pm 6$  cpm and  $1546 \pm 187$  cpm for bFGF (2ng/ml) and aFGF (10ng/ml), respectively.

A)



B)





In contrast, heparin was shown to enhance the binding of both bFGF (Fig. 3.9A) and aFGF (Fig. 3.9B) to the FGFR on heparinase treated cells. Heparin significantly potentiated the binding of aFGF to FGFR at concentrations ranging from 0.001ug/ml to 100ug/ml but had an inhibitory effect on aFGF binding at 1mg/ml. Enhancement of bFGF binding by heparin was less marked than with aFGF and occurred over a narrower concentration range (0.1-1ug/ml), although again at 1mg/ml, heparin was highly inhibitory. This observation that heparin does not enhance the binding of bFGF to its receptor to the same extent as it does aFGF is in agreement with the observation that heparin does not generally potentiate bFGF-induced mitogenesis *in vitro* to the same degree as it does aFGF. This further supports the idea that potentiation of aFGF activity by heparin is related to its ability to enhance aFGF binding to its receptor.

In order to determine whether soluble cell surface HSPGs could enhance aFGF binding like heparin, HSPGs were isolated from both the BALB/c 3T3 cell surface and from the culture medium and assayed for their ability to enhance aFGF binding to the FGFR on cells treated with heparinase just prior to the assay. HSPGs isolated from the cell surface enhanced aFGF binding to FGFR by 42%, 45% and 23% above that observed in the absence of added HSPG on heparinase-treated cells, when used at concentrations of 0.1ug/ml, 0.5ug/ml and 1ug/ml, respectively. HSPGs isolated from the culture medium were more potent, enhancing aFGF binding by 72%, 70% and 43% when used at concentrations of 0.1ug/ml, 1ug/ml and 5ug/ml, respectively. However, these HSPGs were not as potent as heparin which when used at 0.1ug/ml enhanced aFGF binding by 116%.

### **3.2.3. Potentiation of Acidic and Basic FGF Action by Heparin**

Before discussing the data described above it was important to examine the effects of heparin on the mitogenic activity of acidic and basic FGF for the BALB/c 3T3 cells used in this study. Figure 3.10 compares the effect of a

FIGURE 3.9: Effect of different concentrations of heparin on (A) bFGF (20ng/ml) and (B) aFGF (20ng/ml) binding to FGFR on heparinase-treated cells. Values represent means  $\pm$  SEM of triplicate values for bFGF and quadruplicate values for aFGF. Similar results were obtained on 3 separate occasions. Nonspecific binding was <20% of cpm bound.



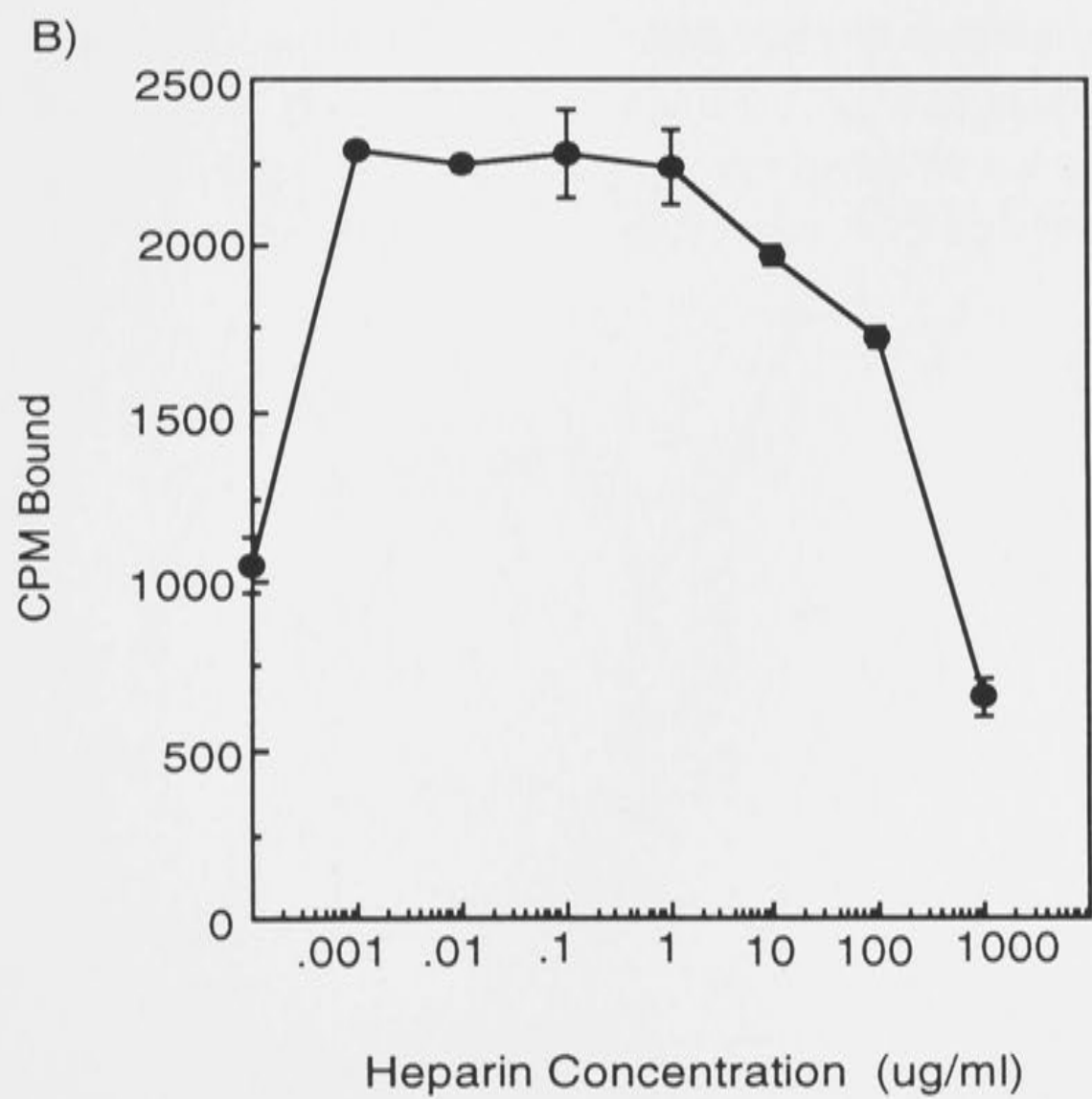
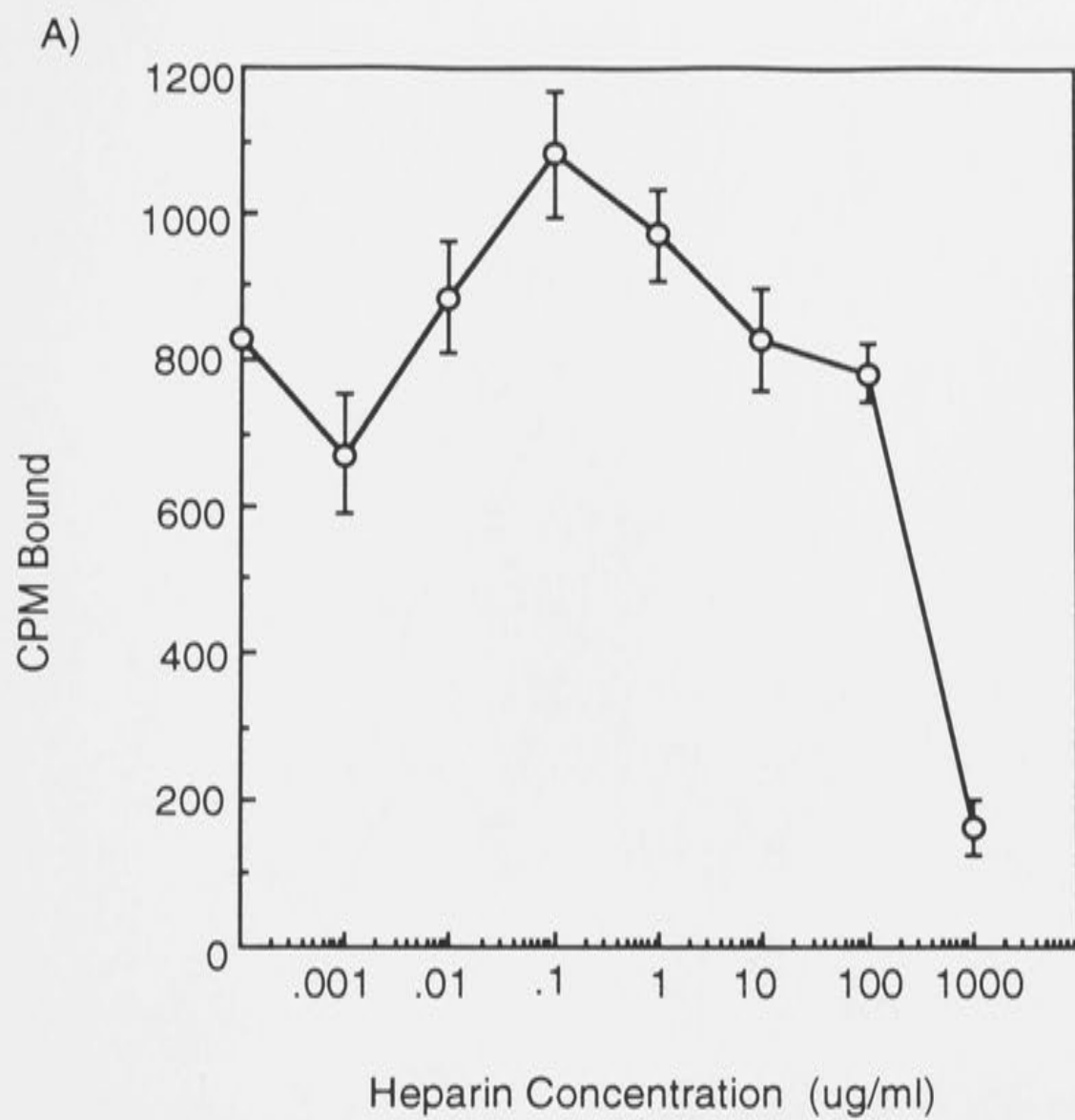
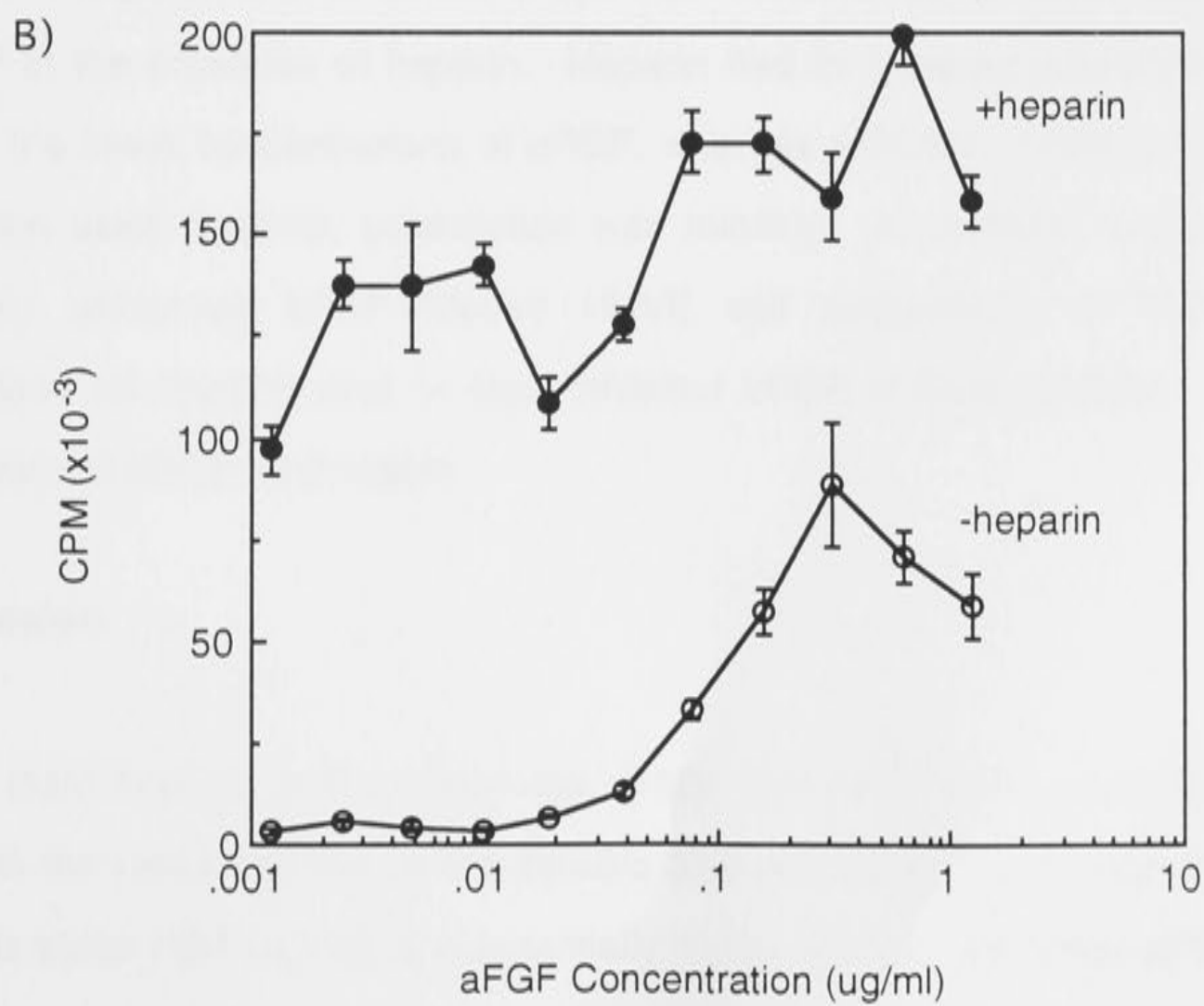
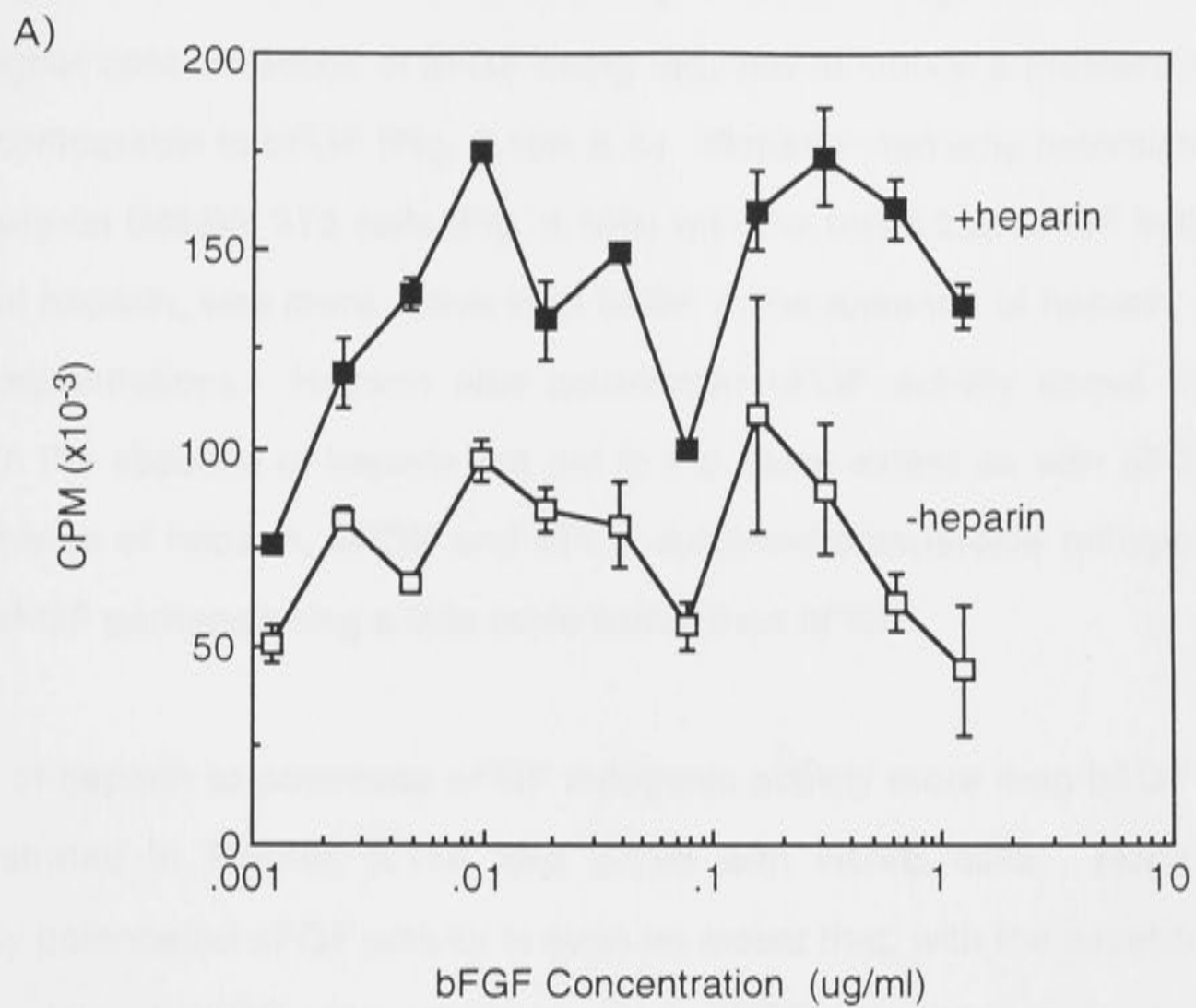


FIGURE 3.10: Effect of heparin (50ug/ml) on (A) bFGF- and on (B) aFGF-induced mitogenesis of BALB/c 3T3 cells.  $^3\text{H}$ -thymidine incorporation is given in cpm  $\times 10^{-3}$ . Values represent mean  $\pm$  SEM of quadruplicate treatments. BALB/c 3T3 mitogenesis in the absence of added heparin gave less than  $4 \times 10^3$  cpm, and in the absence of FGF and heparin gave  $3.7 \times 10^3$  cpm.





constant concentration of heparin (5ug/ml) on acidic and basic FGF-induced mitogenesis of BALB/c 3T3 cells. In the absence of heparin, aFGF was substantially less active than bFGF at inducing 3T3 cell mitogenesis, at least 100-fold higher concentrations of aFGF being required to induce a proliferative response comparable to bFGF (Fig. 3.10A & B). Heparin markedly potentiated aFGF activity on BALB/c 3T3 cells (Fig. 3.10B) with the result that aFGF in the presence of heparin, was more active than bFGF in the absence of heparin, at all FGF concentrations. Heparin also potentiated bFGF activity above that observed in the absence of heparin but not to the same extent as with aFGF. In the presence of heparin, aFGF and bFGF exhibited comparable mitogenic activities, aFGF perhaps being a little more active than bFGF.

The ability of heparin to potentiate aFGF mitogenic activity more than bFGF is further illustrated in Figures 3.11A and 3.11B with HUVE cells. Heparin dramatically potentiated aFGF activity to such an extent that, with the exception of the three lowest aFGF concentrations used, aFGF was more active than bFGF without heparin, and at all but a few FGF concentrations, more active than bFGF in the presence of heparin. Heparin had its greatest potentiating effect with the lower concentrations of aFGF, whereas with the highest aFGF concentration used (1ug/ml), potentiation was minimal. In contrast, heparin only slightly enhanced bFGF-induced HUVE cell mitogenesis at bFGF concentrations  $>0.16\mu\text{g/ml}$  and, in fact, inhibited bFGF mitogenic activity at concentrations of bFGF  $<0.01\mu\text{g/ml}$ .

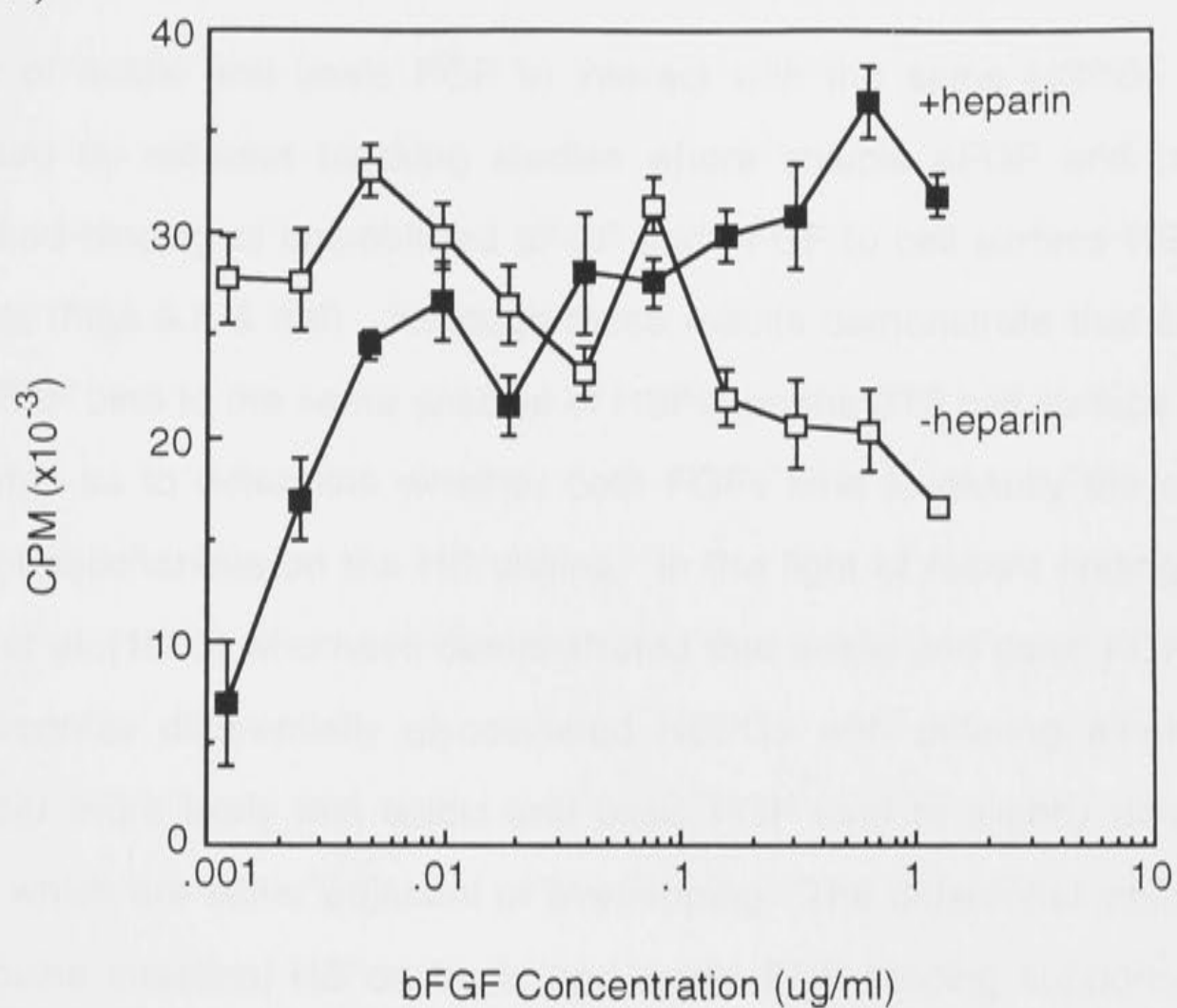
### 3.3. Discussion

The three main findings in this study are, firstly, that acidic and basic FGF interact with the same HSPGs on the BALB/c 3T3 cell surface, secondly, that bFGF binds these HSPGs with a substantially higher affinity than does aFGF

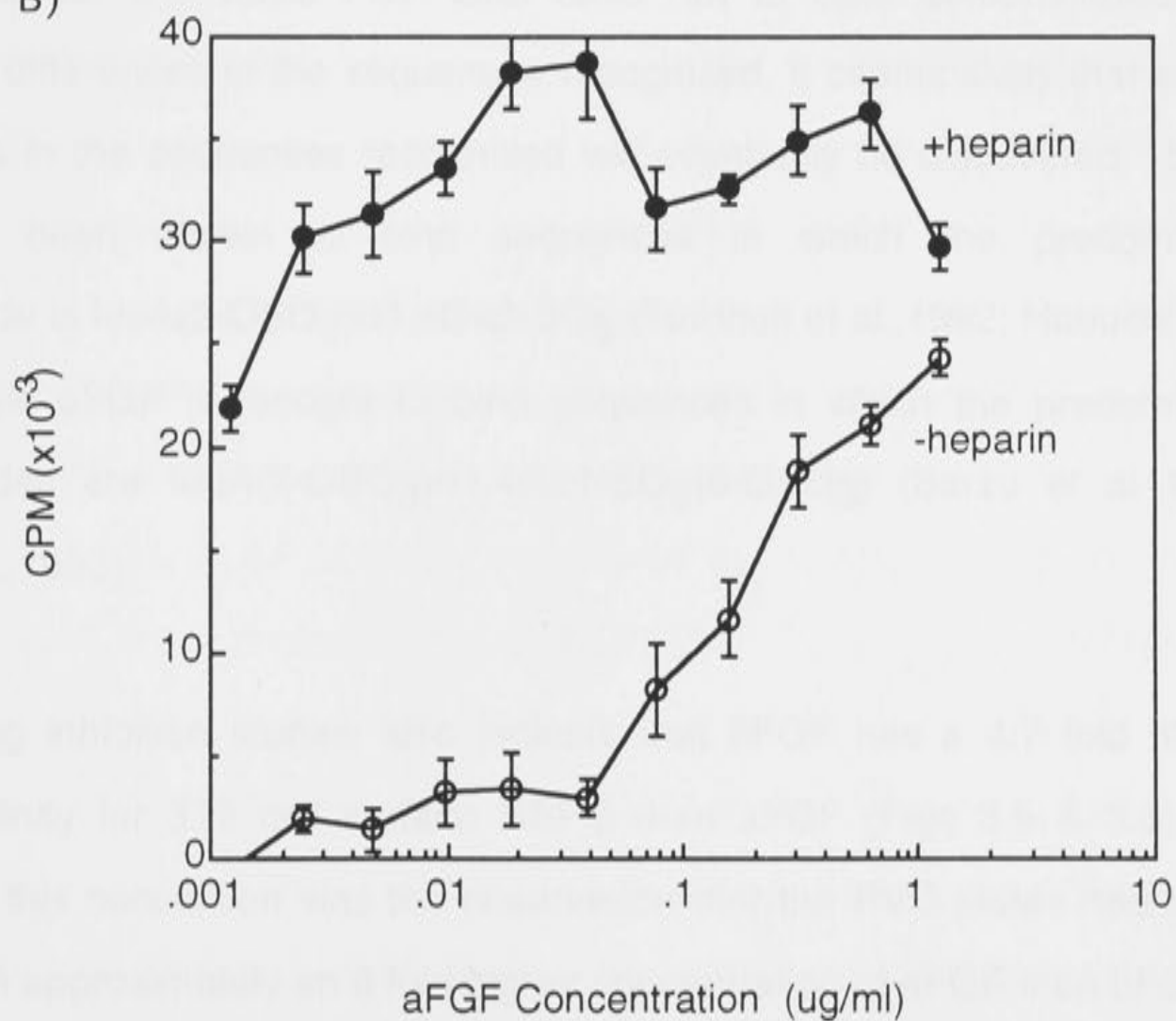


FIGURE 3.11: Effect of heparin (5ug/ml) on (A) bFGF- and on (B) aFGF-induced mitogenesis of HUVE cells.  $^3\text{H}$ -thymidine incorporation is given in cpm  $\times 10^{-3}$ . Values, from which background cpm have been subtracted, represent mean  $\pm$  SEM of quadruplicate treatments.

A)



B)





and thirdly, that cell surface HSPGs stabilize the interaction of bFGF with the FGFR much more effectively than they do aFGF.

The ability of acidic and basic FGF to interact with the same HSPGs was demonstrated by receptor blocking studies where soluble aFGF and bFGF totally blocked binding of immobilized aFGF and bFGF to cell surface HSPGs on 3T3 cells (Figs 3.5 & 3.6). Although these results demonstrate that acidic and basic FGF bind to the same species of HSPG on the 3T3 cell surface they do not enable us to determine whether both FGFs bind to exactly the same sequence of saccharides on the HS chains. In the light of recent findings by Nurcombe et al., (1993) who have demonstrated that acidic and basic FGF are able to recognize differentially glycosylated HSPGs with differing affinity, it would appear more likely that acidic and basic FGF bind to slightly different sequences which are either adjacent or overlapping. The differential inhibitory effect of bovine intestinal HS on basic and acidic FGF binding supports this point (Figs 3.3 & 3.4). Although chemical analysis of the saccharide sequences to which acidic and basic FGF bind have not to date demonstrated any significant differences in the sequences recognized, it seems likely that subtle differences in the sequences recognized will eventually be discovered. Basic FGF has been shown to bind sequences in which the predominant disaccharide is  $\text{IdoA}(2\text{-OSO}_3)\alpha 1,4\text{GlcNSO}_3$  (Turnbull et al., 1992; Habuchi et al 1992), while aFGF is thought to bind sequences in which the predominant disaccharides are  $\text{IdoA}(2\text{-OSO}_3)\alpha 1,4\text{GlcNSO}_3(6\text{-OSO}_3)$  (Barzu et al., 1989; Mach et al., 1993).

The binding inhibition studies also indicate that bFGF has a 4.7 fold higher binding affinity for 3T3 cell surface HSPG than aFGF (Figs 3.5 & 3.6). In support of this conclusion was the observation that the PVC plates had to be coated with approximately an 8 fold higher concentration of aFGF than bFGF to facilitate HSPG-mediated binding of 3T3 cells (Fig. 3.2), despite the fact that



radiolabeled FGFs adhered to the plates equally well. Although it is generally accepted that aFGF has a lower affinity for heparin than does bFGF, their  $K_d$ s being determined as 91nM and 2.2nM respectively (Lee & Lander 1991), the relative affinities of acidic and basic FGF for cell surface HSPGs are less well defined, their  $K_d$ s being reported to lie within the range of 2-10nM (Moscatelli 1987). However, lower salt concentrations have been used to elute aFGF (0.75M) from cell surface HSPG than are used to elute bFGF (>2.0M) suggesting that aFGF exhibits a lower affinity for HSPG than bFGF (Olwin & Rapraeger 1992). On the other hand, Nurcombe et al., (1993) have detected a HSPG during embryonic development which has a higher affinity for acidic than basic FGF. Presumably such a HSPG is not expressed on BALB/c 3T3 cells.

The kinetic studies which compared the rates of dissociation of acidic and basic FGF from FGFR in the presence and absence of endogenous HSPG highlight the difference in affinities of acidic and basic FGF for HSPGs and the importance of HSPG in stabilizing the interaction of FGF with the FGFR. Basic FGF was shown to dissociate from the FGFR >20-fold slower in the presence of HSPGs than in their absence,  $t_{1/2}$ 's being >250min and 12min, respectively (Figs 3.7A & 3.8A). These results are in agreement with those of Nugent and Edelman (1992) who found bFGF to dissociate from FGFR at a 16 fold faster rate in the absence of HSPGs. As Nugent and Edelman (1992) have suggested, HSPG may prolong the period that bFGF is bound to the FGFR thereby enabling receptor activation which might not occur in the absence of HSPGs. In contrast to bFGF, aFGF was found to dissociate relatively rapidly from FGFR both in the presence and absence of HSPG,  $t_{1/2}$  being determined as 25min and 7min, respectively (Figs 3.7B & 3.8B). Thus, these results demonstrate that aFGF dissociates from FGFR in the presence of HSPG at least 10-fold faster than does bFGF. Since this study has shown bFGF has a considerably higher affinity for 3T3 cell surface HSPG than aFGF these results



also suggest that aFGF is not able to utilize 3T3 cell surface HSPGs to form ternary complexes as effectively as does bFGF.

In this study, heparin was shown to potentiate aFGF-induced mitogenesis on both BALB/c 3T3 cells and HUVE cells to a significantly greater extent than it did bFGF-induced mitogenesis (Figs 3.10A & B, 3.11A & B) which confirms the findings of others (Thornton et al., 1983; Schreiber et al., 1985; Lobb et al., 1986; Orlidge & D'Amore 1986). A possible explanation for this potentiation difference may be that aFGF's low affinity for endogenous HSPG on the 3T3 cell surface is not sufficient to ensure maximal binding of aFGF to its receptor. Acidic FGF very likely has a higher affinity for heparin and is able to utilize exogenously added heparin more effectively than endogenous HSPG to promote high affinity binding to the FGFR. In contrast, bFGF has a sufficiently high affinity for cell surface HSPG to enable it to utilize them in high affinity interactions with the FGFR without any requirement for exogenously added heparin.

Unfortunately the data presented in this chapter does not enable me to resolve the issue as to how heparin/HSPG enhance the binding of FGF to the FGFR. The two most likely possibilities are that heparin/HSPG induce a conformational change in the growth factor thus enabling it to bind to the FGFR (Schreiber et al., 1985; Kaplow et al., 1990; Yayon et al., 1991) or that HSPGs are required to stabilize the binding of FGF to its receptor and form a "ternary complex" with them (Nugent & Edelman 1991). These two models are, however, not mutually exclusive and I have presented data in support of both models. In support of the "ternary complex" model, my data strongly suggest that the difference between acidic and basic FGFs' dissociation rates from FGFR is due to their difference in affinity for cell surface HSPGs. Nugent & Edelman (1992) have demonstrated that the difference between "high affinity" binding, traditionally defined as FGF binding to FGFR, and "low affinity" binding, ie., FGF binding to



HSPG, is due only to the slower rate at which bFGF dissociates from the "high affinity" site. Since in their study, bFGF dissociated from FGFR on heparinase-treated cells at a rate 16-fold higher than it did from FGFR in the presence of HSPG, they proposed that the presence of HSPGs is required for a high affinity binding interaction between FGF and FGFR to occur. An HSPG-induced conformational change in the growth factor would not be expected to affect its rate of dissociation from FGFR.

In order for heparin to act as a substitute for HSPG in the formation of a ternary complex, it would have to bind heparin receptors located adjacent to the FGFR or to a heparin-binding domain on the FGFR itself. Indeed potentiation of aFGF-induced mitogenesis by heparin may be the result of the formation of more ternary complexes between aFGF and FGFR than aFGF would form in the presence of HSPG. If this idea is correct, then in the presence of heparin, the rate at which aFGF dissociates from FGFR on heparinase-treated cells would be expected to be lower than in its absence. However, in my system, heparin did not act to decrease the rate at which acidic and basic FGF dissociated from the FGFR. In fact, heparin increased the net amount of acidic and basic FGF bound to the FGFR on heparinase treated cells (Fig. 3.9) without affecting either association (results not shown) or dissociation rates (Fig. 3.8), lending support to the idea of a conformational change. Rather than heparin stabilizing the formation of a ternary complex it may be enhancing the ability of the growth factor to bind to its receptor with the result that more FGFRs are being engaged in the presence of heparin. Certainly, the ability of heparin to promote the binding of FGFs to soluble forms of FGFR in a cell-free system (Ornitz et al., 1992; Ornitz & Leder 1992) strongly suggests that heparin is able to promote FGF binding purely by inducing a conformational change in the growth factor.



One of the difficulties in interpreting the data from my binding assays is that due to the large number of FGFRs on 3T3 cells, it is not possible to distinguish which interactions are those of biological relevance. Such interactions are likely to comprise a small subset of the total number of binding interactions observed in the assay. As the data of Schreiber et al.,(1985) suggests, FGF mitogenic activity requires only partial receptor occupancy. Similarly, it is not known whether the interaction between FGF and FGFR in the soluble receptor assays occurring in the absence of cells, is actually one of biological significance. It is possible that only a small number of receptors need to be engaged to induce a productive signal and that they require the formation of a ternary complex.

Further support for this idea comes from the observation that in my system, higher concentrations of heparin are required to potentiate aFGF-induced mitogenesis than are required to potentiate aFGF binding to heparinase-treated cells. Perhaps this can be explained in part by the likelihood that heparin receptors on the BALB/c 3T3 cell surface outnumber FGFRs by a factor of at least 300:1 (see chapter 4) and thus many of the heparin receptors would not be located in a position favourable for the formation of ternary complexes with FGF and the FGFR. Thus a large proportion of the heparin/FGF complexes formed would be sequestered by heparin receptors distant from the FGFR. Yet the formation of a productive complex may require that FGF is bound either to HSPG which is predominantly cell surface bound, or to heparin bound to heparin receptors and perhaps relatively few of these ternary complexes are needed to produce sufficient activation of receptors. Moreover, the possibility that heparin is stabilizing the interaction between FGF and FGFR by binding to a putative heparin-binding domain on the FGFR has not yet been considered (see chapter 4). If such an interaction was occurring then it would tend to support the idea that heparin promotes the formation of a ternary complex.



### 3.4. Summary

Heparan sulfate proteoglycans (HSPGs) on the cell surface act as low affinity binding sites for acidic and basic FGF (Moscatelli 1987) and play an important role in the interaction of FGF with the FGF receptor (FGFR). In this study, several aspects of the interaction of FGFs with HSPGs were examined. First, reciprocal cross blocking studies demonstrated that aFGF and bFGF bind to identical or closely associated HS motifs on BALB/c 3T3 cell surface HSPGs. However, the binding affinity of the two growth factors for these HSPGs differs considerably, binding-inhibition data indicating that aFGF has a 4.7 fold lower affinity than bFGF for 3T3 HSPG. Subsequent studies of dissociation kinetics demonstrated that bFGF dissociates from the FGFR at least 10-fold slower than aFGF ( $t_{1/2}$  >250min versus 25 min) whereas, following removal of cell surface HSPGs by heparinase treatment, the dissociation rate of both FGFs is similar and rapid ( $t_{1/2}$  12min versus 7min). These results support the concept that cell surface HSPGs stabilize the interaction of FGFs with FGFR, possibly by the formation of a ternary complex, with the lower binding affinity of aFGF for HSPG, compared with bFGF for HSPG, resulting in binding to FGFR being less effectively stabilized by HSPG. Such a model may explain the lower mitogenic activity of aFGF compared with bFGF for 3T3 cells and suggests that heparin usually potentiates aFGF-induced, but not bFGF-induced, mitogenesis because of aFGF's relatively low affinity for cell surface HSPGs. In this context, heparin had no effect on the rate at which acidic and basic FGF dissociated from FGFR but it did enhance the net binding of aFGF and bFGF to FGFR on cells from which HSPGs had been removed by treatment with heparinase. These data are discussed in the context of the ability of heparin/HSPGs to promote FGF binding to FGFR by inducing a conformational change in the growth factor and/or by enabling the formation of a ternary complex.



## Chapter 4

### **Evidence that Carboxyl-Reduced Heparin Fails to Potentiate aFGF Activity via an Inability to Interact with Cell Surface Heparin Receptors**

#### **4.1. Introduction**

The potentiation of aFGF activity by heparin was first observed by Thornton et al.,(1983) who reported that heparin greatly potentiated the mitogenic effect of endothelial cell growth factor (ECGF), a semi-purified aFGF preparation, on the proliferation of endothelial cells. They found that maximal growth could be induced by 25ug/ml of ECGF in the presence of heparin, but that 100-200ug/ml of ECGF was required to support significant growth in the absence of heparin. Heparin alone had no effect on endothelial cell replication, indicating that both factors were required for optimum growth. It was therefore suggested that heparin-like substances may play an important role in regulating cell growth in normal and injured vessels (Thornton et al.,1983). Ten years later, although cell surface HSPGs and heparin have been clearly implicated in the regulation of FGF-induced cell growth, and significant advances have been made towards understanding the mechanisms involved, it is still not known exactly how heparin potentiates the mitotic (Thornton et al.,1983), chemotactic (Terranova et al 1985), neurotrophic (Unsicker et al.,1987) and angiogenic (Lobb et al.,1985) activities of aFGF.

A major aim of my Ph.D. project was to gain a better understanding of how heparin potentiates aFGF-induced mitogenesis of BALB/c 3T3 cells. Of particular interest in this regard was the demonstration by Belford et al.,(1992) that a chemically modified form of native heparin which has had the majority of its carboxyl groups reduced, is able to bind aFGF as effectively as does the native heparin molecule but is less potent in augmenting aFGF-induced mitogenesis of 3T3 and HUVE cells. The ability of the carboxyl-reduced



heparin (CR-heparin) to bind aFGF but not to potentiate aFGF activity as well as native heparin, suggests that potentiation is not simply due to the ability of heparin to bind with high affinity to the growth factor. Based on these observations, experiments presented in this chapter are directed towards testing the hypothesis that potentiation of aFGF activity by heparin is due to the crosslinking of the FGFR and a cell surface heparin-specific receptor with an aFGF/heparin complex. Models proposed to explain the mechanism of potentiation to date, have not considered the possible involvement of cell surface heparin receptors in stabilizing the aFGF/heparin complex on the FGFR. However, saturable binding sites for heparin and HS have been shown to exist on various cell types (Castellot 1985; Vannuchi et al., 1986, 1988; Barzu et al., 1986a,b; Redini et al., 1989). Instead, the models have proposed that potentiation by heparin is due to heparin's ability to protect aFGF from proteolysis by degradative enzymes, thereby extending its half-life (Damon et al., 1989) and/or its ability upon binding to aFGF to produce a conformational change in the growth factor, resulting in its increased affinity for the FGFR (Schreiber et al., 1985; Kaplow et al., 1990; Yayon et al., 1991). A detailed discussion of these models is presented in section 1.8 of this thesis. This chapter describes a detailed comparison of the effects of native and CR-heparin on aFGF action and provides evidence that the failure of CR-heparin to potentiate aFGF activity is probably due to its reduced ability to bind to heparin binding sites either on the FGFR or on adjacent heparin binding molecules on the cell surface.

## **4.2. Results**

### **4.2.1. General Properties of Carboxyl-Reduced Heparin**

The procedure used routinely to prepare carboxyl-reduced preparations of heparin (Taylor et al., 1976) only reduces a proportion of the carboxyl groups of the molecule. In our hands, a single reductive treatment resulted in 35-40% of



the heparin carboxyl groups remaining in the non-reduced form whereas two and three rounds of reduction resulted in 25% and 17%, respectively, of the carboxyl groups being non-reduced (Table 4.1). However, when these three CR-heparin preparations were compared for their ability to compete for the binding of aFGF to heparin coupled agarose beads, the CR-heparin with 35-40% carboxyl groups remaining, competed almost as effectively as native heparin whereas the more highly reduced preparations were much less effective competitors (Table 4.1). These differences in competitive activity were not due to differences in sulfate content as the CR-heparin preparations were indistinguishable from native heparin in their content of sulfate (data not shown).

When tested for their ability to enhance aFGF-induced mitogenesis, the two most highly reduced heparin preparations exhibited negligible potentiating activity, ie., maximum of 2-3 fold increase in proliferation above aFGF alone (Table 4.1). Similarly, the CR-heparin with 35-40% carboxyl groups remaining was a relatively poor potentiator of aFGF mitogenesis compared with native heparin (Table 4.1), despite apparently binding to aFGF with high affinity. These results confirm the earlier findings of Belford et al.,(1992) and also demonstrate that the carboxyl group content of heparin plays a critical role in its interaction with aFGF. The preparation of CR-heparin with 35-40% of carboxyl groups remaining was selected for the subsequent studies.

#### **4.2.2. Potentiation of aFGF Activity by Heparin and CR-Heparin**

Initial studies compared the ability of heparin and CR-heparin to potentiate aFGF mitogenic activity in more detail. The ability of heparin and CR-heparin to potentiate aFGF-induced mitogenesis of BALB/c 3T3 fibroblasts over a period of four days is shown in Figure 4.1. Potentiation of aFGF activity by heparin did not take effect until after the first 24 hours of incubation and peaked at the end of the second day, decreasing steadily thereafter, as did aFGF

Table 4.1: Ability of Different CR-Heparin Preparations to Bind to aFGF and Potentiate aFGF Mitogenic Activity

Rounds of Carboxyl Group Reduction	Carboxyl Group Content (% untreated)	Percent Inhibition of aFGF Binding to Heparin <sup>a</sup>	Potentiation of aFGF Mitogenic Activity		
			Concentration (ug/ml)	<sup>3</sup> H-Thymidine Incorporation cpm <sup>b</sup>	Potentiation Factor <sup>c</sup>
Nil	100	90	10	13,882±562	9.9
			50	44,348±4212	31.7
x1	35-40	76	10	2,455±270	1.8
			50	9,144±1293	6.5
x2	25	0	10	4,068±302	2.9
			50	3,473±283	2.5
x3	17	15	10	3,204±813	2.3
			50	1,389±232	1.0

<sup>a</sup>Percent inhibition of binding of 10ug/ml of aFGF to heparin-agarose beads in the presence of 500ug/ml of each heparin preparation.

<sup>b</sup> The data are mean ± SEM of triplicate treatments, [<sup>3</sup>H]thymidine incorporation being measured 24-48 hours following culture initiation. Acidic FGF was used at a concentration of 10ng/ml and in the absence of heparin gave 1,400 ± 579 cpm. Background mitogenesis occurring in the absence of aFGF and heparin has been subtracted from all values.

<sup>c</sup>Increase in [<sup>3</sup>H]thymidine incorporation over cultures containing 10ng/ml of aFGF in the absence of heparin.



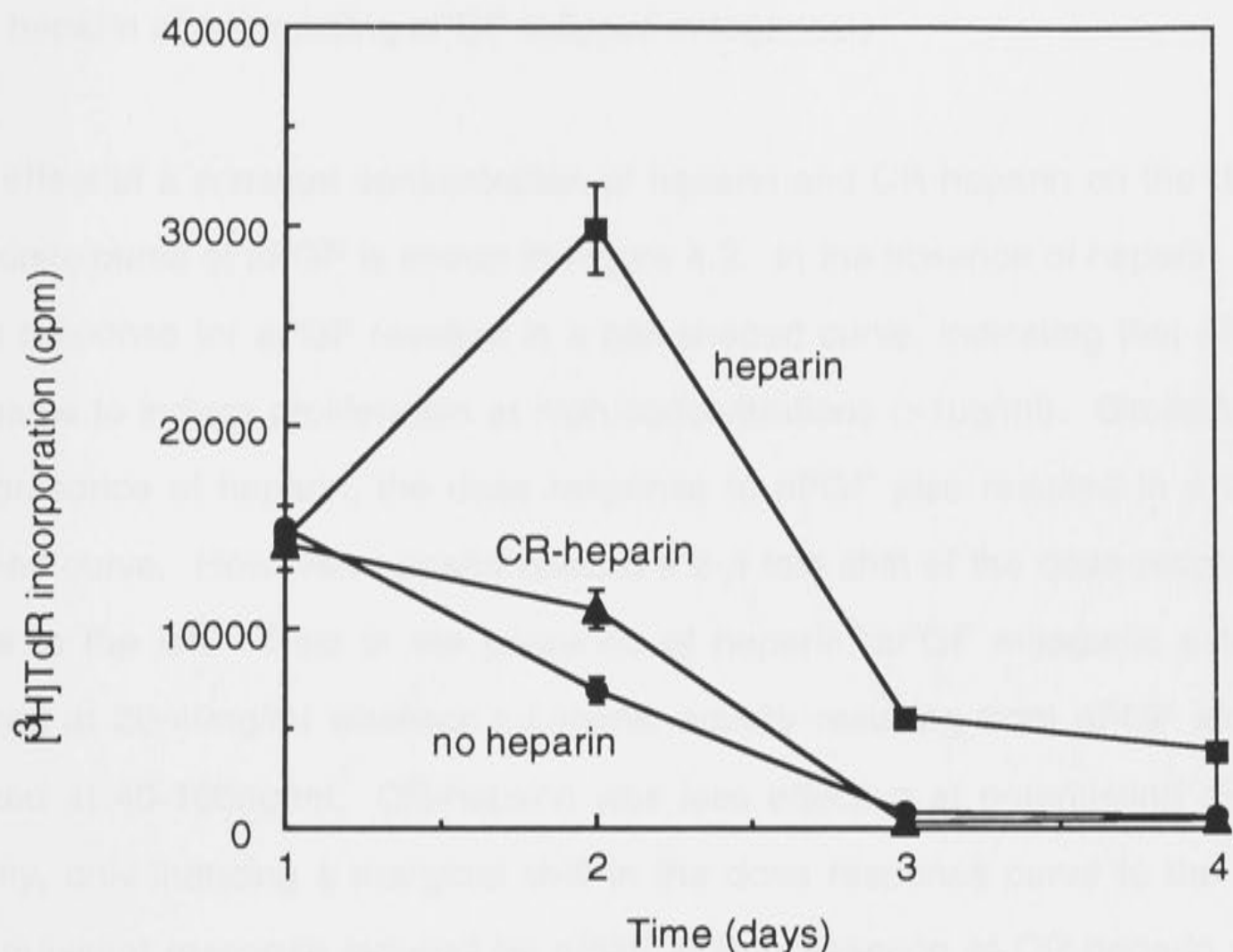


FIGURE 4.1: Time course showing the proliferative response of BALB/ c 3T3 cells to aFGF (10ng/ml) in the presence of heparin (■), CR-heparin (▲) or in the absence (●) of heparin. Both forms of heparin were used at 50ug/ml and alone gave no background proliferation. [ $^3\text{H}$ ]TdR was added for the final 24 hours of each time point and its incorporation is expressed as cpm. Values represent means  $\pm$  SEM (n=4) of one representative experiment. Background cpm, subtracted from each time point, were <2700 cpm.

activity in the absence of heparin. CR-heparin was virtually inactive at the concentration used, showing negligible potentiation of aFGF activity for the duration of the time course. Thus this experiment confirms the results of Belford et al.,(1992) who showed that CR-heparin was consistently less potent than heparin at augmenting aFGF-induced mitogenesis.

The effect of a constant concentration of heparin and CR-heparin on the dose response curve of aFGF is shown in Figure 4.2. In the absence of heparin, the dose response for aFGF resulted in a bell shaped curve, indicating that aFGF is unable to induce proliferation at high concentrations ( $>1\mu\text{g/ml}$ ). Similarly, in the presence of heparin, the dose response to aFGF also resulted in a bell-shaped curve. However, heparin caused a 2-3 fold shift of the dose response curve to the left. Thus in the presence of heparin, aFGF mitogenic activity peaked at 20-40ng/ml whereas mitogenic activity resulting from aFGF alone, peaked at 40-160ng/ml. CR-heparin was less effective at potentiating aFGF activity, only inducing a marginal shift in the dose response curve to the left. The maximal response induced by aFGF in the presence of CR-heparin was slightly higher than that seen with heparin but this was not a consistent observation in other experiments. In agreement with the results of the time course experiment (Fig. 4.1.), the activity of aFGF when used at a concentration of 0.01 $\mu\text{g/ml}$  was not potentiated by CR-heparin but was potentiated by heparin. Although in this experiment both heparins were used at 5 $\mu\text{g/ml}$ , similar dose response curves were obtained when heparin and CR-heparin were used at concentrations as high as 50 $\mu\text{g/ml}$ . This data is also consistent with earlier studies by Belford et al.,(1992) using CR-heparin.

#### **4.2.3. Binding Affinities of Heparin and CR-Heparin for Acidic and Basic FGF**

Figure 4.3 shows the relative abilities of heparin and CR-heparin to bind to bFGF (A) and aFGF (B). Scatchard analysis of the binding data found heparin and CR-heparin to have similar binding affinities for bFGF although CR-



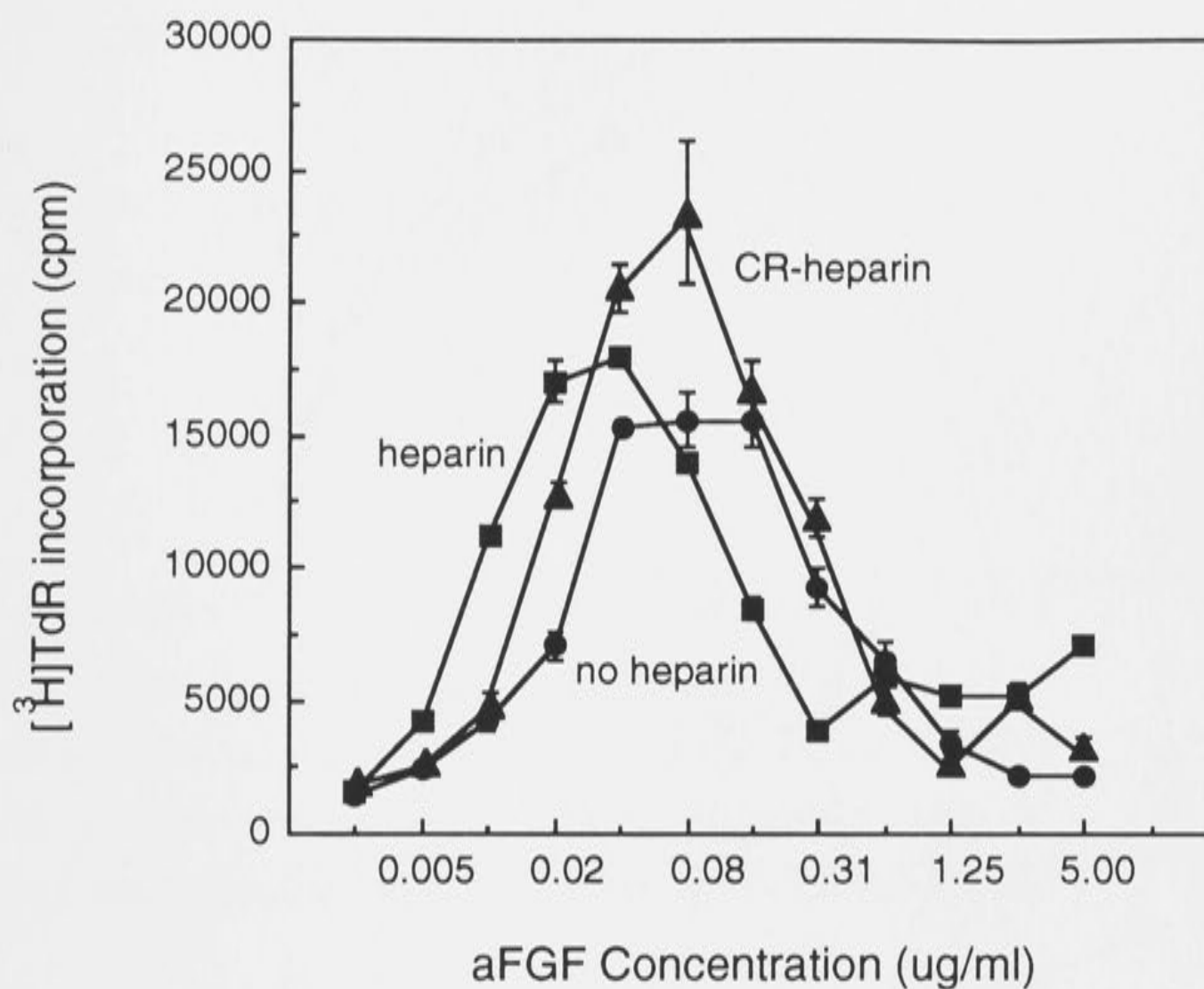
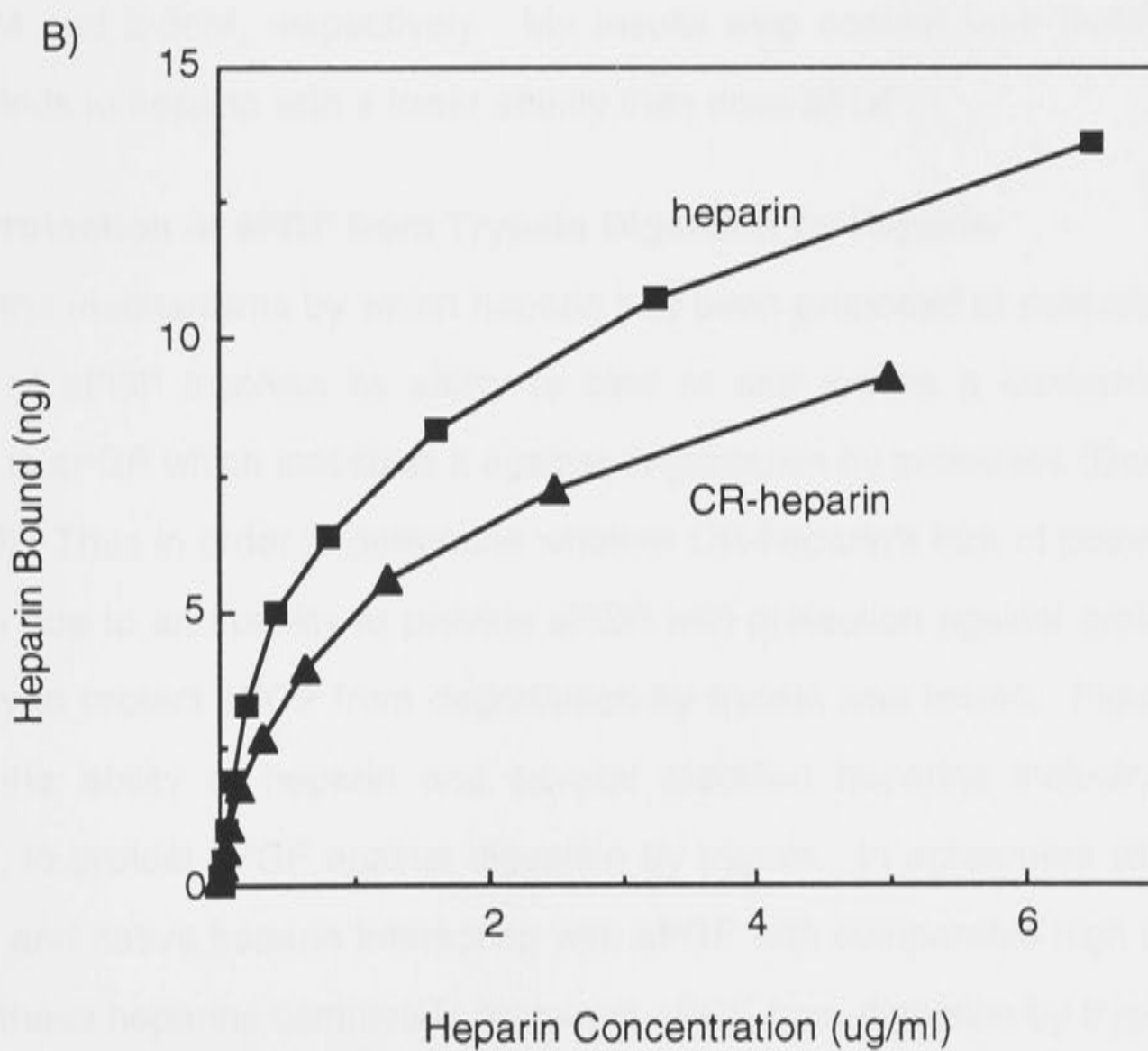
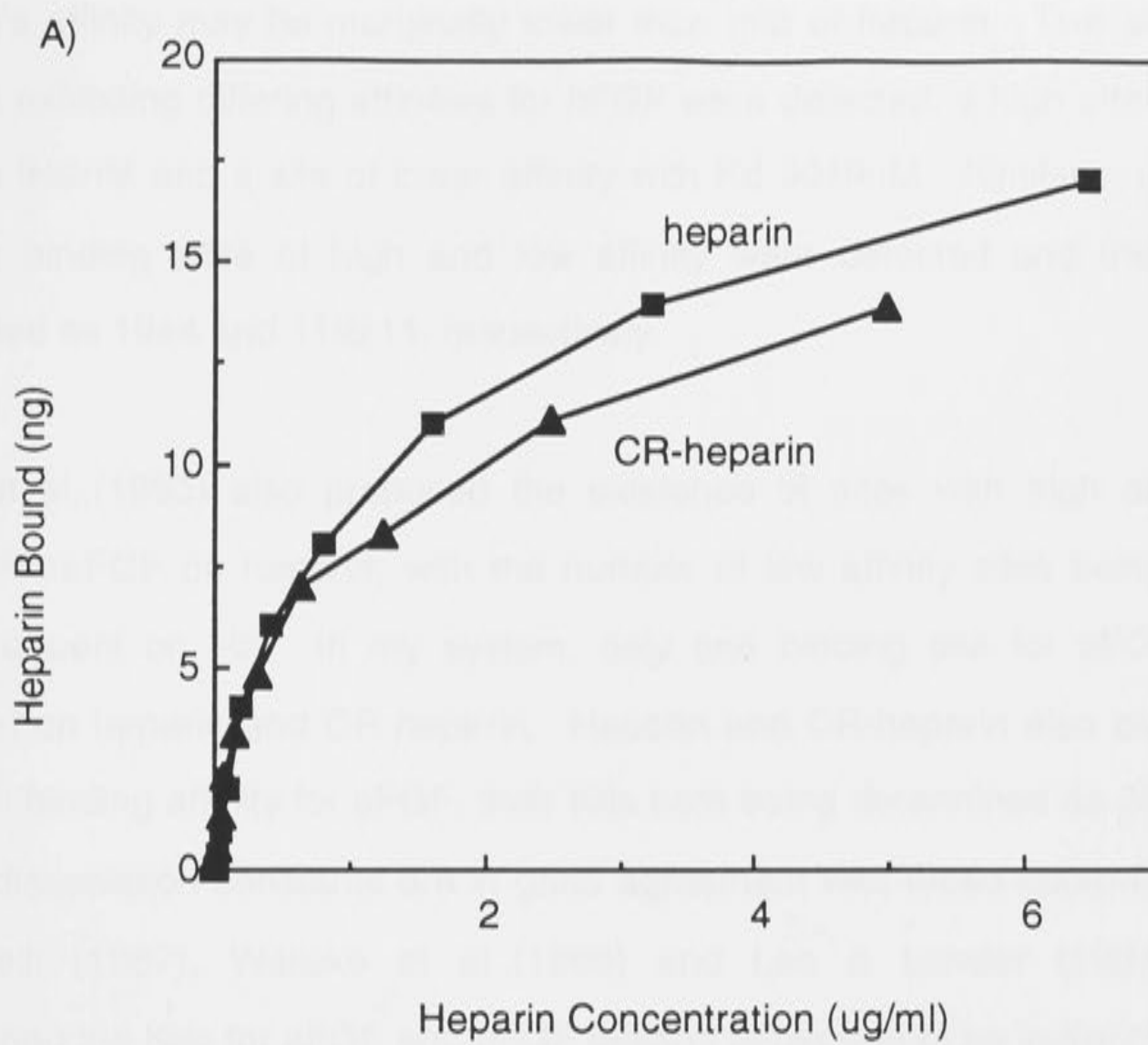


FIGURE 4.2: Proliferative response of BALB/c 3T3 cells to varying concentrations of aFGF in the presence of a constant concentration (5ug/ml) of heparin (■), CR-heparin (▲) or in the absence (●) of heparin. Incubation was for 48 hours and [ $^3\text{H}$ ]TdR was added for the second 24 hours of the incubation. Heparin and CR-heparin alone gave no proliferation above the background of 1700cpm. Values represent means  $\pm$  SEM (n=4) of one representative experiment.

FIGURE 4.3: Comparison of the ability of  $^{125}\text{I}$ -heparin (■) and  $^{125}\text{I}$ -CR-heparin (▲) to bind to immobilized A) bFGF and B) aFGF. Data points represent specific binding and are the means of quadruplicate treatments. Non-specific binding in the presence of a 100 fold excess of unlabeled heparin was <10%.





heparin's affinity may be marginally lower than that of heparin. Two sites on heparin exhibiting differing affinities for bFGF were detected, a high affinity site with  $K_d$   $9 \pm 2$  nM and a site of lower affinity with  $K_d$   $90 \pm 9$  nM. Similarly, on CR-heparin binding sites of high and low affinity were detected and their  $K_d$ s calculated as  $19 \pm 4$  and  $119 \pm 11$ , respectively.

Mach et al.,(1993) also proposed the existence of sites with high and low affinity for aFGF on heparin, with the number of low affinity sites being even more frequent on HS. In my system, only one binding site for aFGF was detected on heparin and CR-heparin. Heparin and CR-heparin also exhibited identical binding affinity for aFGF, their  $K_d$ s both being determined as  $35 \pm 5$  nM. These dissociation constants are in good agreement with those determined by Moscatelli (1987), Walicke et al.,(1989) and Lee & Lander (1991) who determined the  $K_d$ s for aFGF and bFGF binding to heparin to be in the range of 60-90 nM and 2-3 nM, respectively. My results also confirm their finding that aFGF binds to heparin with a lower affinity than does bFGF.

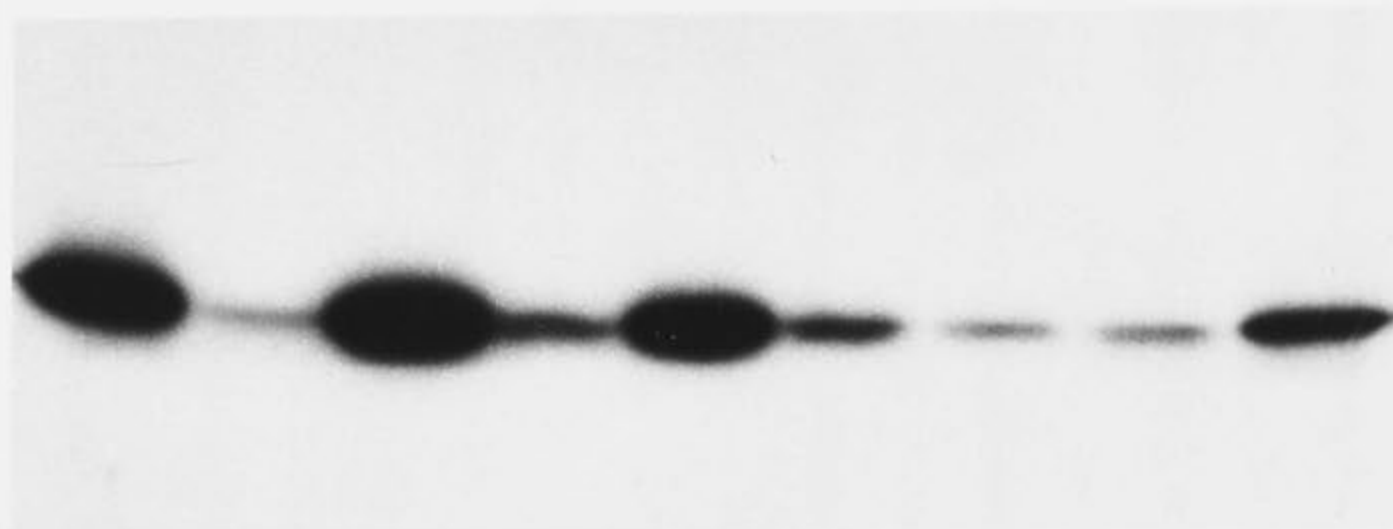
#### **4.2.4. Protection of aFGF from Trypsin Digestion by Heparin**

One of the mechanisms by which heparin has been proposed to potentiate the activity of aFGF involves its ability to bind to and induce a conformational change in aFGF which stabilizes it against degradation by proteases (Damon et al.,1989). Thus in order to determine whether CR-heparin's lack of potentiating ability is due to an inability to provide aFGF with protection against proteases, its ability to protect aFGF from degradation by trypsin was tested. Figure 4.4. shows the ability of heparin and several modified heparins including CR-heparin, to protect aFGF against digestion by trypsin. In agreement with CR-heparin and native heparin interacting with aFGF with comparable high affinity, both of these heparins completely protected aFGF from digestion by trypsin. In contrast, N-desulfated/ acetylated heparin, N-desulfated/acetoacetylated heparin and N-desulfated heparin offered only a small degree of protection



FIGURE 4.4: Ability of native heparin and several chemically modified heparins (1mg/ml) to protect  $^{125}\text{I}$ -aFGF (1.8ng) from digestion by trypsin (96ug/ml). Mixtures were incubated overnight at 37°C and then run on a 11% SDS-PAGE gel and radiolabeled aFGF detected by autoradiography.

			<i>native</i>	<i>N-desulfated acetylated carboxyl-reduced</i>	<i>N-desulfated</i>	<i>N- &amp; O-desulfated</i>	<i>N- &amp; O-desulfated, N-resulfated</i>	<i>N-desulfated acetoacetylated</i>
aFGF	+	+	+	+	+	+	+	+
Trypsin	-	+	+	+	+	+	+	+





while N- & O-desulfated/N-resulfated heparin and N- & O-desulfated heparin gave no protection. This is largely in agreement with the ability of these heparins to bind aFGF as it has been shown previously (Belford et al., 1992) that N-desulfated, N- & O-desulfated/N-resulfated, and N- & O-desulfated heparins do not bind aFGF while N-desulfated/acetylated heparin and N-desulfated/acetoacetylated heparin exhibit some affinity for aFGF. These results suggest that the ability of heparin to potentiate aFGF activity cannot be fully explained by its ability to protect aFGF from proteolysis.

#### **4.2.5. Binding of Radiolabeled aFGF to Heparinase Treated Cells**

It has also been suggested that heparin potentiates aFGF-induced activity by inducing aFGF to assume a conformation with increased affinity for its receptor (Schreiber et al., 1985; Kaplow et al., 1990). Thus, in the next series of experiments, the ability of either heparin or CR-heparin to enhance the binding of aFGF to its receptor was tested. In order to examine FGFR binding of aFGF without the participation of cell surface HSPGs, BALB/c 3T3 cells were pretreated with heparinase to remove surface HSPGs. Figure 4.5 shows the effect of different concentrations of heparin and CR-heparin on the binding of  $^{125}\text{I}$ -aFGF to heparinase treated cells. Heparin at concentrations ranging between 1ng/ml and 100ug/ml significantly enhanced the binding of  $^{125}\text{I}$ -aFGF to the FGFR. However, at high concentrations (1mg/ml), heparin inhibited the binding of aFGF to its receptor. CR-heparin also significantly enhanced aFGF binding to the FGFR but to a much lesser extent than did heparin and over a narrower concentration range, namely, between 1ng/ml and 1ug/ml. Concentrations of CR-heparin greater than 10ug/ml inhibited aFGF binding. These data indicate that CR-heparin is a poor potentiator of aFGF binding to FGFR compared with heparin.

#### **4.2.6. Binding of Heparin and CR-Heparin to Cell Surface Receptors**

If heparin enhances the binding of aFGF to its receptor by forming a complex with aFGF and crosslinking both an FGFR and a heparin receptor then it is

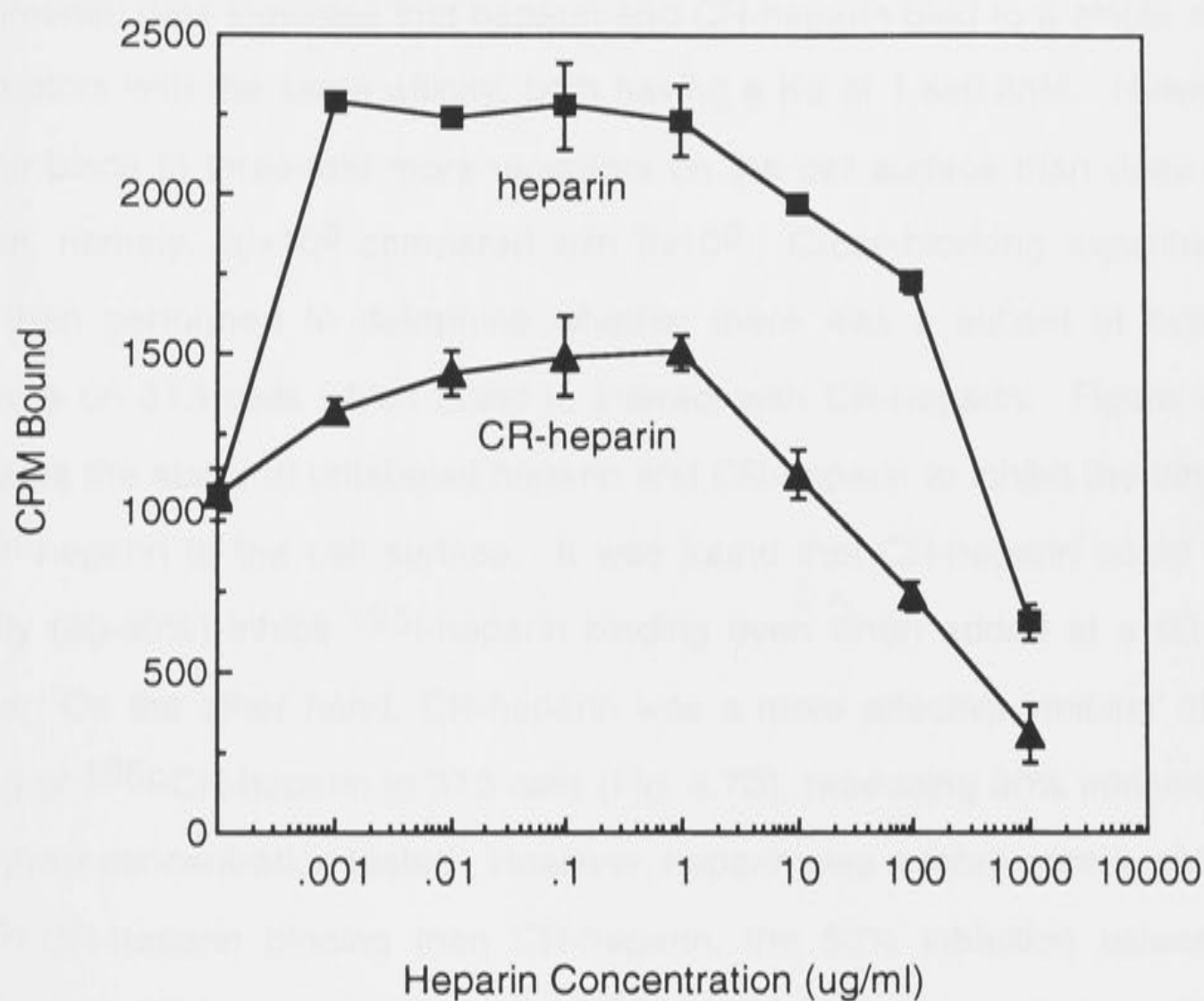


FIGURE 4.5: Effect of different concentrations of heparin (■) and CR-heparin (▲) on the binding of  $^{125}\text{I}$ -aFGF (20ng/ml) to heparinase treated BALB/c 3T3 cells. Values represent means  $\pm$  SEM (n=4) of one representative experiment. Similar results were obtained on 3 separate occasions. Values represent specific binding. Non-specific binding in the presence of an excess of unlabeled aFGF (50ug/ml) was routinely between 5 and 10% of cpm bound.



conceivable that the poor potentiating activity of CR-heparin may be the result of an inability to interact with heparin receptors on the cell surface. Figure 4.6 demonstrates that both heparin and CR-heparin bind to the surface of BALB/c 3T3 cells in a specific and saturable manner. Scatchard analysis of the experimental data indicates that heparin and CR-heparin bind to a single class of receptors with the same affinity, both having a  $K_d$  of  $1.8 \pm 0.2 \text{ nM}$ . However, heparin binds to three-fold more receptors on the cell surface than does CR-heparin, namely,  $25 \times 10^6$  compared with  $8 \times 10^6$ . Cross-blocking experiments were then performed to determine whether there was a subset of heparin receptors on 3T3 cells which failed to interact with CR-heparin. Figure 4.7A compares the ability of unlabeled heparin and CR-heparin to inhibit the binding of  $^{125}\text{I}$ -heparin to the cell surface. It was found that CR-heparin could only partially (30-40%) inhibit  $^{125}\text{I}$ -heparin binding even when added at a 50 fold excess. On the other hand, CR-heparin was a more effective inhibitor of the binding of  $^{125}\text{I}$ -CR-heparin to 3T3 cells (Fig. 4.7B), producing 80% inhibition at the highest concentration tested. However, heparin was a more potent inhibitor of  $^{125}\text{I}$ -CR-heparin binding than CR-heparin, the 50% inhibition values for heparin and CR-heparin being 0.1mg/ml and 1.2mg/ml, respectively. These results demonstrate that CR-heparin only binds to a subset of cell surface receptors specific for native heparin and is not recognized by a specific subset of cell surface receptors which do not interact with heparin. Thus, the poor ability of CR-heparin to potentiate aFGF-induced activity may be related to its inability to bind to certain heparin receptors on the cell surface.

#### **4.2.7. Effect of Heparin and CR-Heparin on the Binding of aFGF and bFGF to Soluble FGFR**

To further clarify the effect of heparin and CR-heparin on the binding of aFGF to the FGFR, some experiments were performed in collaboration with Dr David Ornitz, St Louis, MO, using a soluble recombinant form of murine FGF receptor (FR1AP). Figure 4.8 compares the ability of heparin and CR-heparin to enhance the binding of aFGF to this receptor in the absence of other cell-

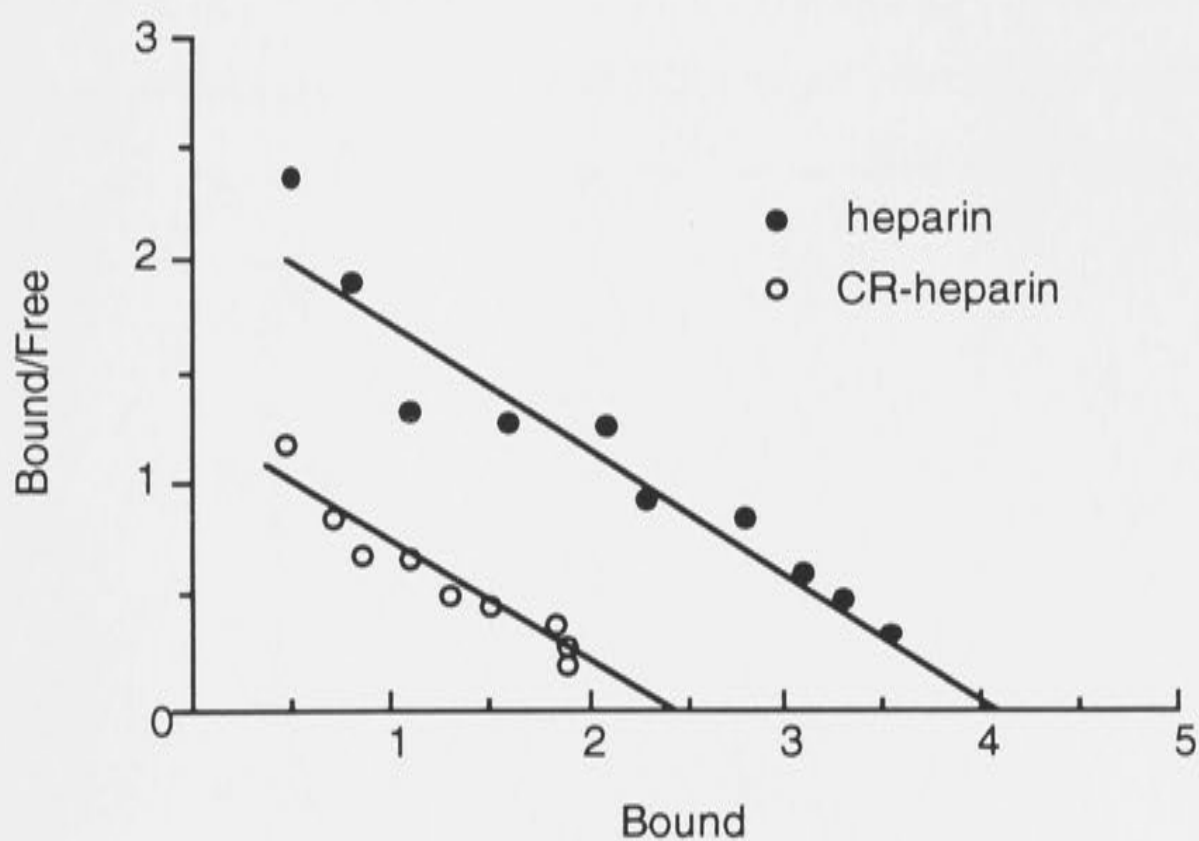
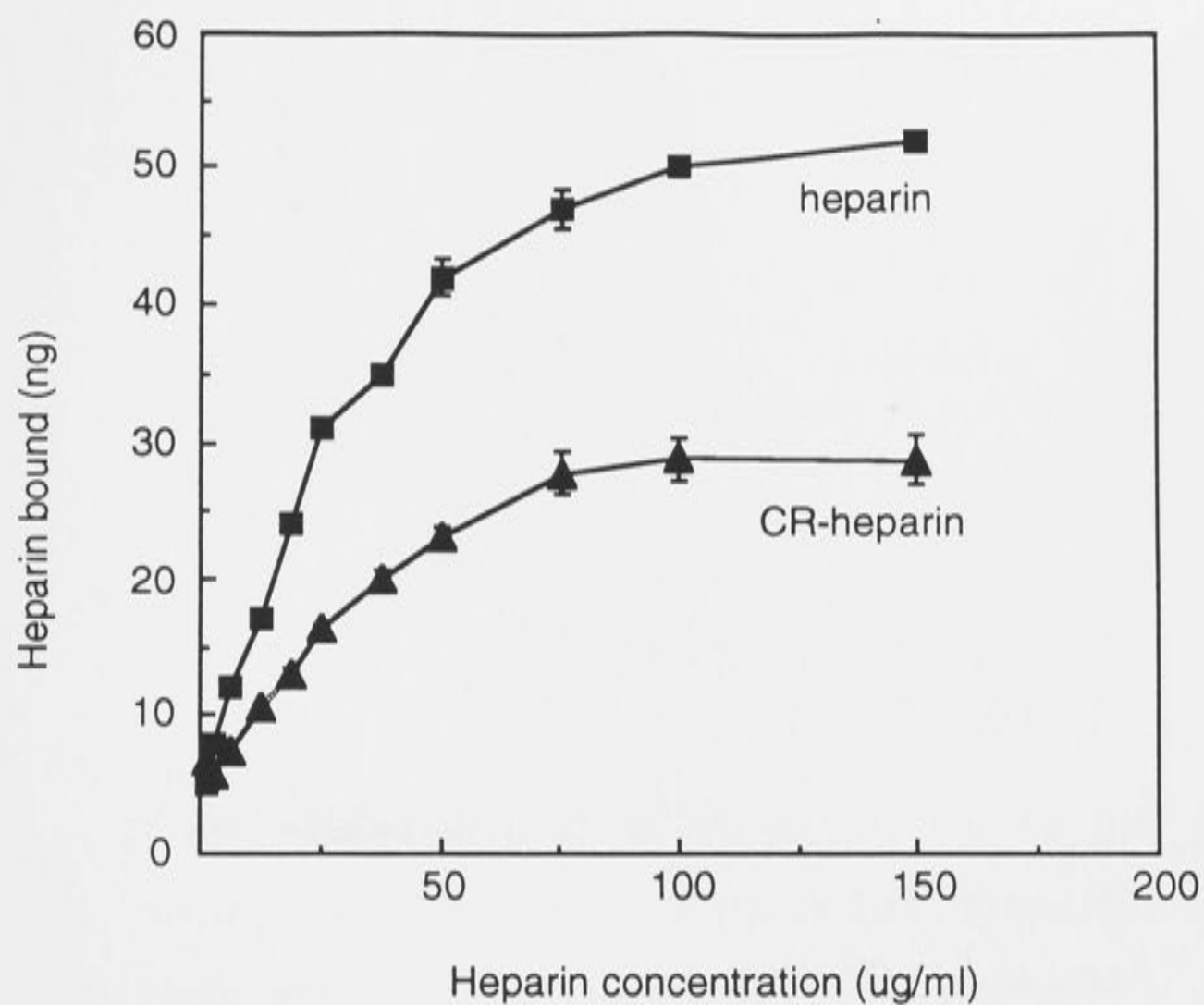


FIGURE 4.6: (A) Specific binding of  $^{125}\text{I}$ -heparin (■) and  $^{125}\text{I}$ -CR-heparin (▲) to BALB/c 3T3 cells. Nonspecific binding (20%) was determined in the presence of a 50-fold excess of unlabeled heparin and subtracted from the total binding. Values represent means  $\pm$  SEM ( $n=4$ ) of one representative experiment. (B) Scatchard plot of experimental data.



FIGURE 4.7: Comparison of the ability of unlabeled heparin (■) and CR-heparin (▲) to inhibit the binding of a constant concentration (100ug/ml) of either  $^{125}\text{I}$ -heparin (A) or  $^{125}\text{I}$ -CR-heparin (B) to BALB/c 3T3 cells. Values represent means  $\pm$  SEM (n=3) and are given as percent of labeled heparin/CR-heparin which was bound in the absence of cold competitor .

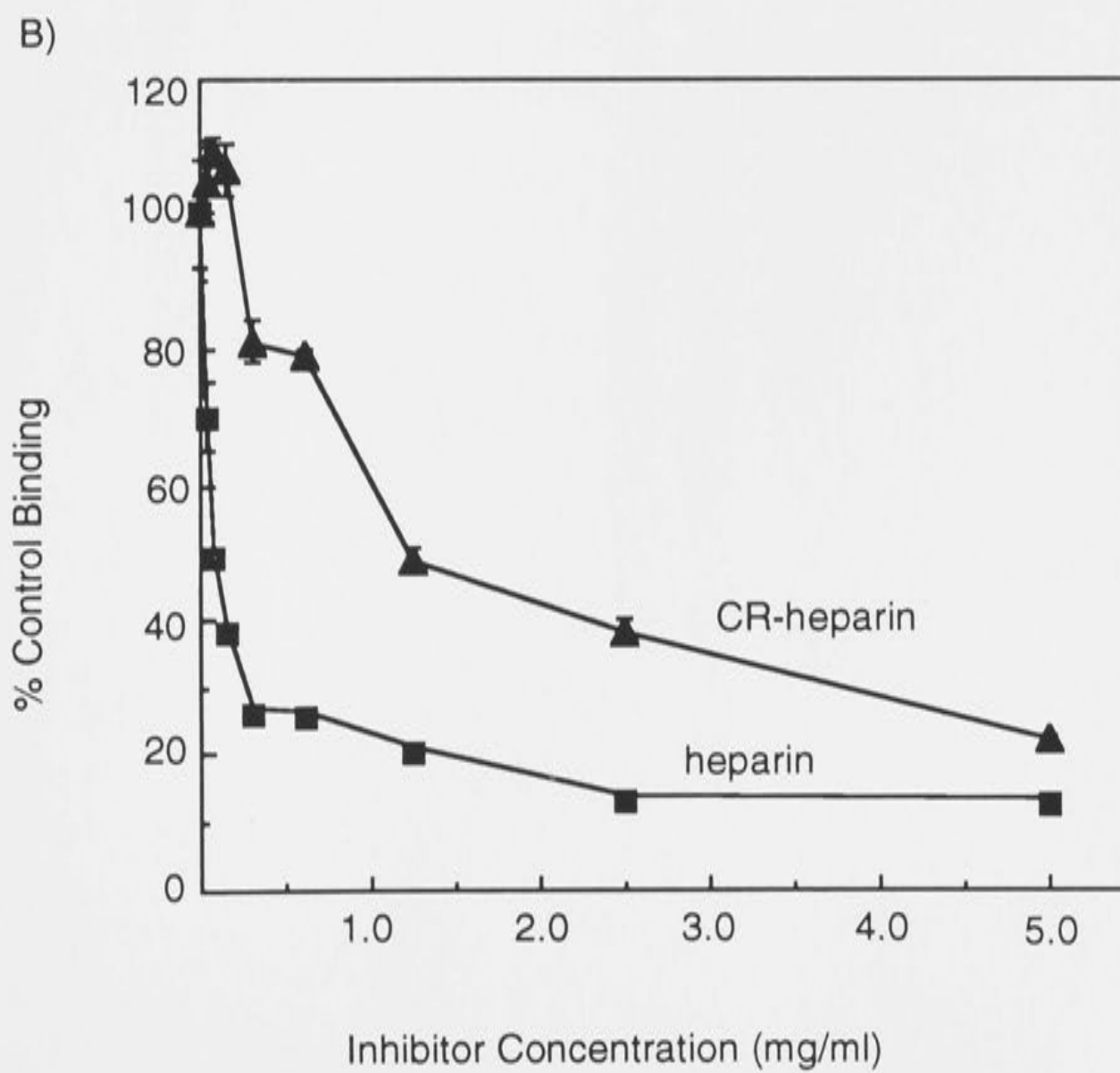
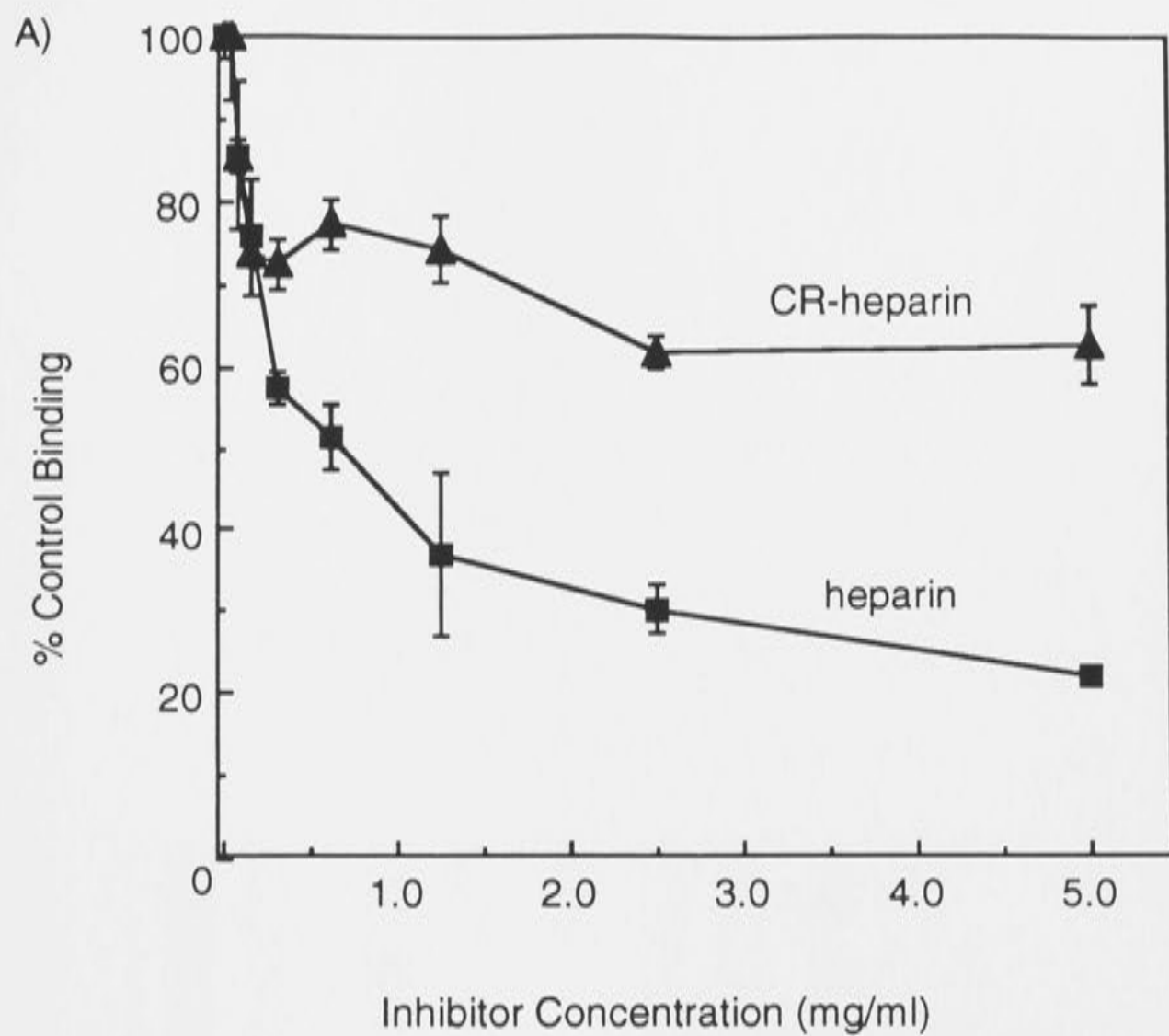
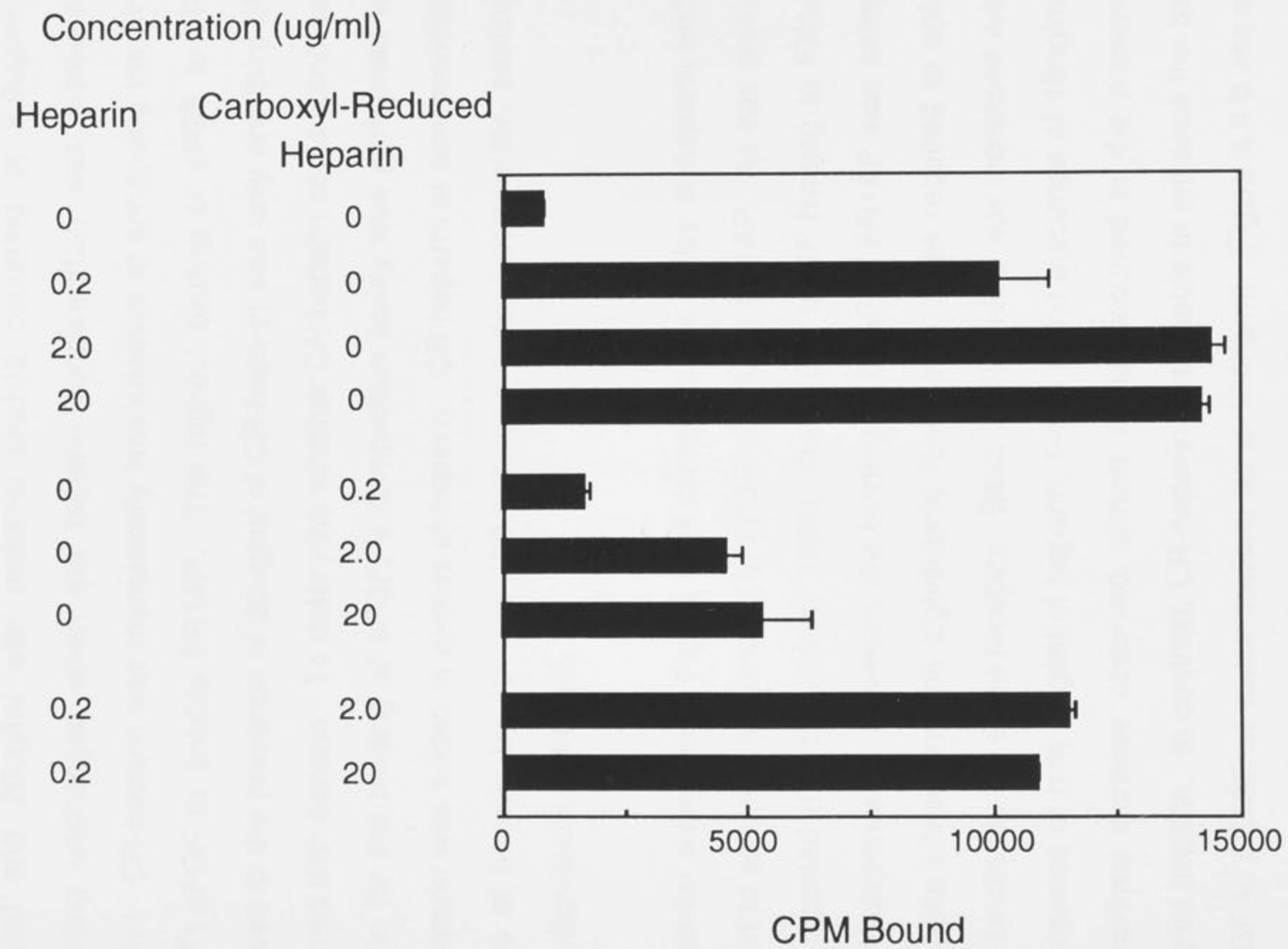




FIGURE 4.8: The effect of different concentrations of heparin and CR-heparin on the binding of  $^{125}\text{I}$ -aFGF to a soluble form of FGFR1. Each bar represents the mean  $\pm$  SD of cpm bound.





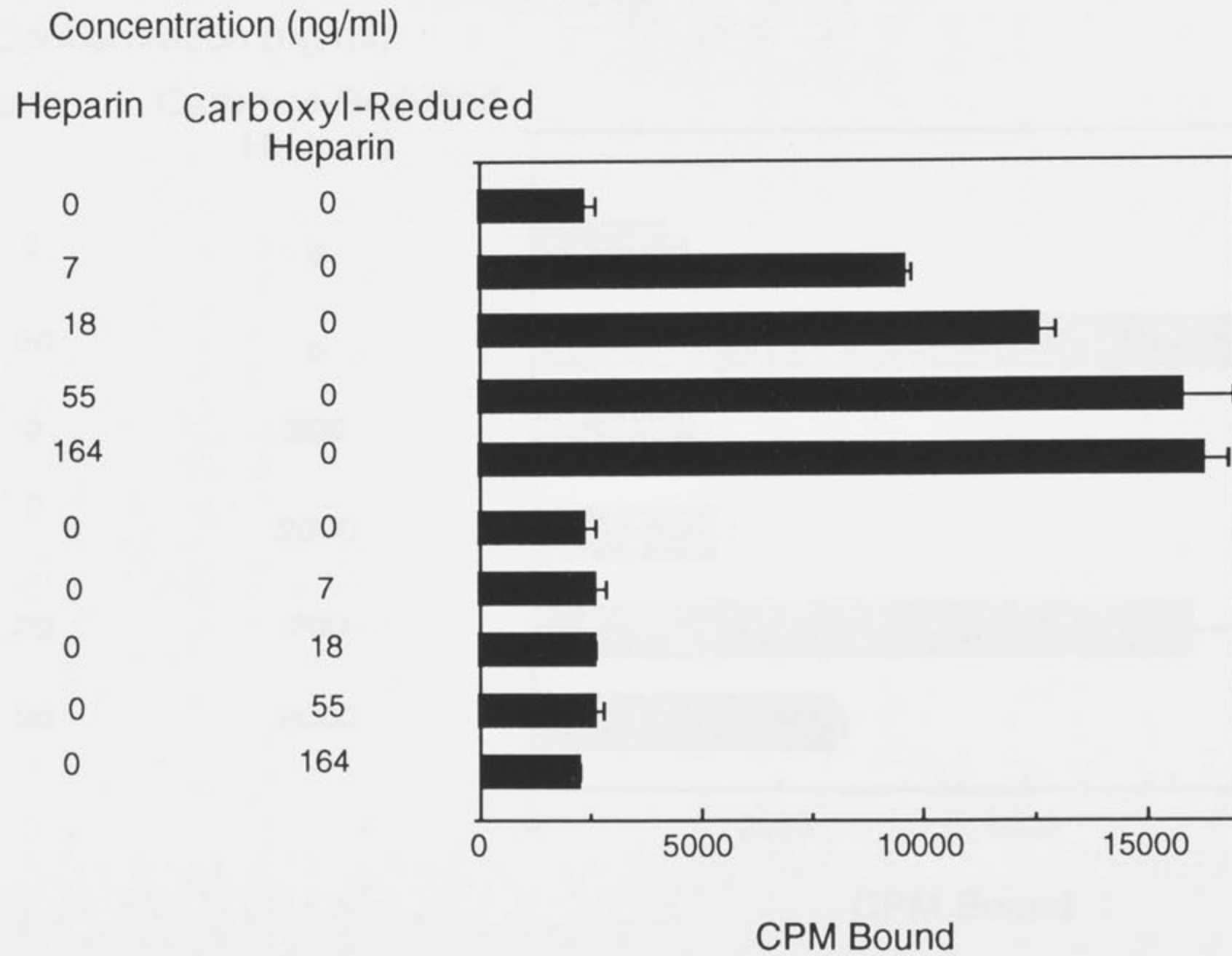
associated molecules. In the absence of heparin or CR-heparin, very little specific binding of aFGF to FR1AP was observed. The binding of  $^{125}\text{I}$ -aFGF to FR1AP was significantly enhanced by heparin at concentrations of 0.2ug/ml, 2.0ug/ml and 20ug/ml with maximal binding occurring at 2.0ug/ml and remaining unchanged when the heparin concentration was increased to 20ug/ml. CR-heparin was dramatically less effective at enhancing the binding of  $^{125}\text{I}$ -aFGF to soluble FR1AP. The highest binding of aFGF to FR1AP achieved in the presence of 20ug/ml of CR-heparin was only one-third of that observed with heparin. To determine whether CR-heparin could compete with heparin for the binding of aFGF, a competition assay was performed where CR-heparin was added in excess of heparin. CR-heparin at concentrations in excess of heparin by 10- and 100-fold, was a poor competitor, binding not being significantly effected.

In a similar experiment, Figure 4.9A compares the ability of heparin and CR-heparin to enhance the binding of bFGF to soluble FR1AP. As has previously been reported (Ornitz & Leder 1992), like aFGF, bFGF binding to FR1AP is heparin-dependent. However, the binding of bFGF to FR1AP was enhanced by heparin concentrations significantly lower than those required to enhance aFGF binding to the same receptor. Basic FGF binding was increased 4-fold in the presence of only 1ng/ml of heparin, 5-fold in the presence of 18ng/ml and the maximal increase observed, almost 7-fold, occurred in the presence of 164ng/ml heparin. In contrast, CR-heparin was unable to enhance the binding of bFGF to FR1AP at concentrations up to 164ng/ml. Figure 4.9.B shows the effect of a 10-fold and 100-fold excess of CR-heparin over heparin on the binding of bFGF to FR1AP. A 10-fold excess of CR-heparin resulted in approximately 18% inhibition of bFGF binding whereas a 100-fold excess resulted in approximately 74% inhibition of heparin-induced binding. Interestingly, CR-heparin is much less effective at enhancing the binding of bFGF to FR1AP than it is at enhancing aFGF binding, but it inhibits bFGF

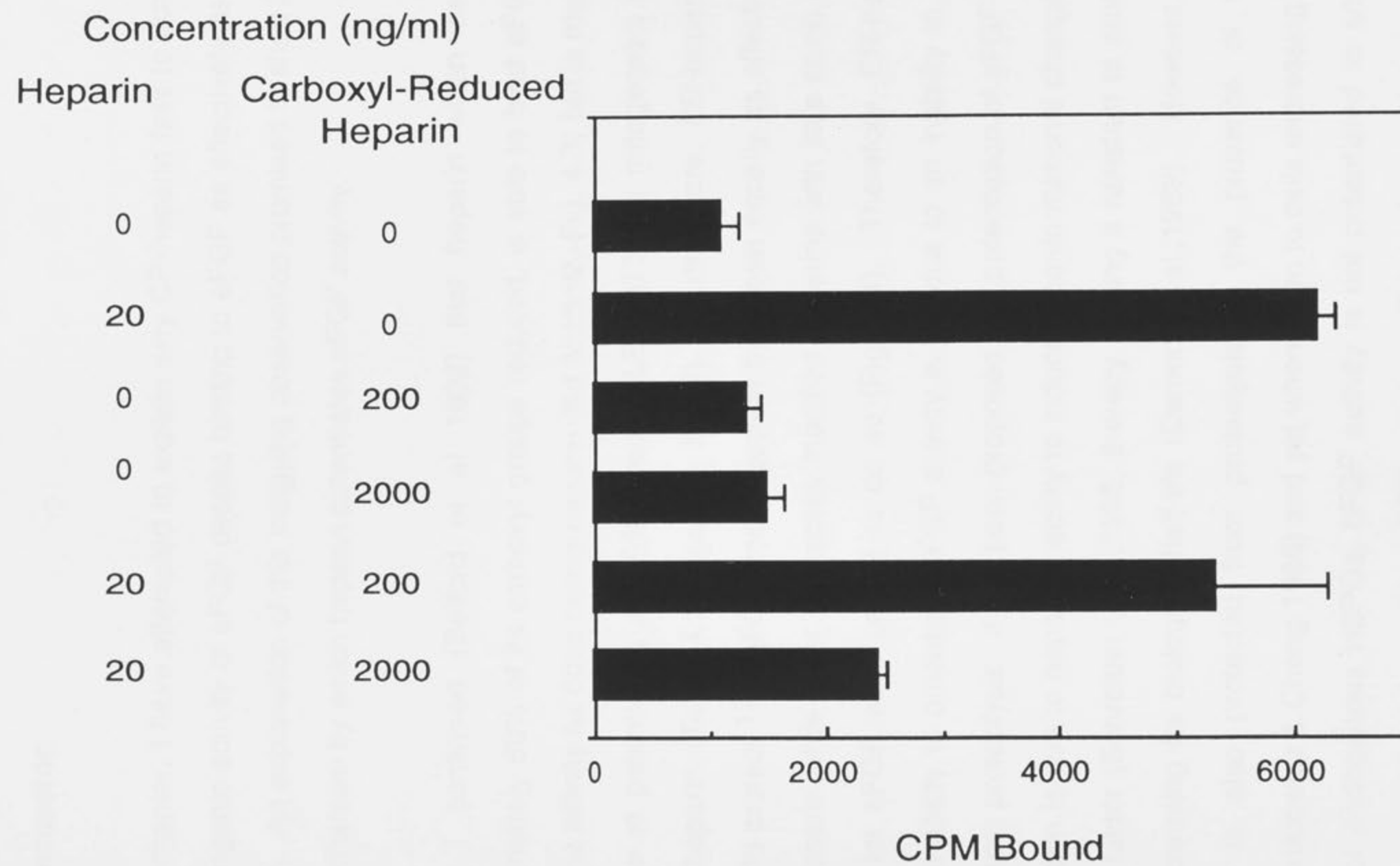
FIGURE 4.9: The effect of different concentrations of heparin and CR-heparin on the binding of bFGF to a soluble form of FGFR1. Figure 4.9.(A) shows the effect of different concentrations of either heparin or CR-heparin on the binding of  $^{125}\text{I}$ -bFGF to FGFR1 while, B) shows the combined effects of heparin and CR-heparin on  $^{125}\text{I}$ -bFGF binding when CR-heparin is at a 10- and 100-fold higher concentration than heparin. Each bar represents the mean  $\pm$  SD of cpm bound.



A)



B)





binding in a mixed competition assay more effectively than it does aFGF binding.

### 4.3. Discussion

In this chapter, I have attempted to explain why CR-heparin fails to potentiate the mitogenic activity of aFGF despite binding to aFGF as effectively as native heparin. An explanation of this intriguing observation promised to shed light on the mechanism by which heparin potentiates aFGF activity.

Initially I confirmed (Belford et al 1992) that heparin which has had approximately 60% of its carboxyl groups reduced, is able to bind aFGF with the same affinity as does native heparin ( $K_d$   $35 \pm 5$  nM, Fig. 4.3) but is much less effective at potentiating aFGF-induced BALB/c 3T3 cell mitogenesis than is native heparin (Table 4.1, Figs 4.1 & 4.2). Furthermore, CR-heparin was shown to protect  $^{125}$ I-aFGF from digestion by trypsin equally as effectively as does heparin while other chemically modified heparins with little or no binding affinity for aFGF were unable to do so (Fig. 4.4). Therefore, CR-heparin's reduced ability to potentiate aFGF activity is not due to an inability to protect aFGF from proteolysis. It has been proposed that potentiation of aFGF activity by heparin is due to heparin's ability to induce a conformational change in the growth factor (Schreiber et al., 1985) thereby making it resistant to proteolysis and increasing its biological half-life (Damon et al., 1989). However, bFGF activity is also protected from proteolysis in the presence of heparin (Gospodarowicz & Cheng 1986) and yet when given to cells expressing normal levels of endogenous HSPGs, bFGF activity is not potentiated to anywhere near the same extent as is aFGF activity. This, taken together with the observation that CR-heparin also protects aFGF from proteolysis, strongly suggests that potentiation of aFGF activity by heparin is not due to heparin's ability to protect aFGF from proteolytic degradation.



On the other hand, heparin and CR-heparin differed in their ability to enhance the binding of aFGF to the FGFR on cells lacking cell surface HSPGs (Fig. 4.5). At low concentrations, heparin significantly enhanced the binding of aFGF to the FGFR on heparinase-treated cells while CR-heparin induced only a small enhancement. Similarly, the soluble FGFR assay demonstrated that CR-heparin is substantially less effective than heparin at enhancing the binding of aFGF to mFR1 (Fig. 4.8). Thus, the data suggest that CR-heparin's poor ability to potentiate aFGF-induced mitogenic activity is due to its failure to effectively enhance the binding of aFGF to its cell surface receptor. I will now discuss possible reasons why this is so.

There appear to be two ways by which CR-heparin may prevent aFGF from forming a stable interaction with its receptor. First, CR-heparin may fail to produce the correct conformational change in the growth factor which is thought to be necessary for FGF binding to its receptor, or second, it may not allow the formation of a stable ternary complex between aFGF and its receptor. The ability of CR-heparin to bind aFGF equally as well as does heparin and to protect aFGF from digestion by trypsin, makes it unlikely that CR-heparin is unable to induce a conformational change in the molecule, although this cannot be totally ruled out (Fig. 1.8). It seems more plausible that CR-heparin fails to enable the formation of a stable ternary complex. A ternary complex could result from an aFGF/heparin complex either crosslinking a cell surface receptor specific for heparin with the FGFR or crosslinking heparin-binding and aFGF-binding sites on the same FGFR (Fig. 1.9). This latter possibility has become an interesting alternative following the recent demonstration that human FGFR1 possesses a heparin-binding site which is essential for FGF binding to FGFR1 (Kan et al., 1993).



The demonstration that CR-heparin is only able to bind to 1/3 the number of heparin receptors on the cell surface to which heparin binds (Figs 4.6 & 4.7) suggests that CR-heparin may be less able to form ternary complexes with aFGF, FGFR and heparin receptors as can occur with heparin. It is possible that there are specific heparin receptors to which CR-heparin does not bind that may be critical for the formation of such ternary complexes with FGFR. Furthermore, the reduced ability of CR-heparin to enhance the binding of aFGF to FGFR, both on heparinase-treated cells and in the soluble receptor binding assay, suggests that CR-heparin may have a low binding affinity for the proposed heparin-binding domain on the FGFR. This would prevent the formation of stable ternary complexes between an aFGF-heparin complex and the FGF and heparin-binding sites on the FGFR.

Preliminary results from Dr Ornitz's laboratory (personal communication) do indeed suggest that CR-heparin has a considerably lower affinity than heparin for the heparin binding site on FGFR. In the absence of FGF, CR-heparin was at least 10 fold less able to bind to the soluble form of murine FGFR1 and approximately 25 fold less able to bind to FGFR3, compared with heparin. Interestingly, while CR-heparin is partially able to enhance the binding of aFGF to FGFR1 in the soluble receptor binding assay, it is totally unable to enhance the binding of bFGF to the same receptor. Since CR-heparin was found to bind aFGF and bFGF equally as well as does native heparin, this inactivity is not due to an inability to bind the growth factors. Furthermore, CR-heparin when in excess of heparin by 100-fold was able to inhibit heparin-induced bFGF binding by approximately 80% whereas it was a much poorer competitor for aFGF binding. These results suggest that acidic and basic FGF have different requirements for binding to FGFR1.

In order to explain the soluble FGFR data two assumptions must be made. First, in order for aFGF to bind to FGFR1 it must undergo a heparin-induced



conformational change whereas with bFGF no such conformational change is required. The second assumption made is that CR-heparin does not bind to the heparin-binding site of the FGFR and this is supported by preliminary data outlined above. Thus, it can be proposed that for optimal binding of aFGF to the FGFR to occur, not only must aFGF bind to heparin and undergo a conformational change which allows it to bind to the receptor binding site on FGFR but the aFGF/heparin complex must also form a ternary complex with the heparin- and FGF-binding sites of FGFR to ensure that a stable receptor-ligand interaction occurs. In contrast, in order for bFGF to bind to FGFR it must form a ternary complex with heparin, the heparin-binding site on FGFR and the growth factor-binding site on the FGFR. It does not, however, require a heparin-induced conformational change in order to bind to the FGFR. The low level of aFGF binding to the FGFR seen in the presence of CR-heparin may be due to the ability of CR-heparin to induce a partial conformational change in aFGF which enables some weak binding to the FGFR. In contrast, the absence of any binding of bFGF to the FGFR is due to CR-heparin's inability to bind to the heparin-binding site of the FGFR and form a ternary complex with it and the growth factor. The relative balance of conformational change versus ternary complex formation may vary with each FGFR and between FGFs with each FGFR. In summary, in order to explain my experimental results it is proposed that for optimal binding of aFGF to FGFR1 to occur, a heparin induced conformational change in aFGF must be followed by the formation of a ternary complex, whereas for the binding of bFGF to FGFR1, only the formation of a ternary complex is required.

Preliminary results from David Ornitz's laboratory (personal communication) using BAF3 cells transfected with mFR1, show that CR-heparin does result in some enhancement of bFGF-induced mitogenesis but not as much as with native heparin which indicates that other cell surface molecules may be involved in the formation of ternary complexes. It is plausible that CR-heparin,



although unable to bind to the heparin-binding site on FGFRs, may utilize cell-surface heparin receptors in the formation of a ternary complex between FGF and FGFR.

An interesting additional observation was that high concentrations of both heparin and CR-heparin inhibited the binding of aFGF to FGFR on heparinase-treated cells. Similar observations have been reported by others (Barzu et al., 1989; Ornitz et al., 1992; Nurcombe et al., 1993). There was however, generally no inhibition of aFGF-induced mitogenic activity by high concentrations of heparin even when cell surface HSPGs were removed by culturing the cells in chlorate (results not shown). This phenomenon remains largely unexplained. Ornitz et al., (1992) have suggested that high concentrations of heparin may inhibit the dimerization of FGF which they suggest is necessary for receptor oligomerization and activation. Alternatively, it is possible that at high concentrations, heparin free of aFGF masks heparin binding sites on the cell surface or on FGFR and thereby inhibits ternary complex formation.

A second interesting observation which deserves comment was that heparin-mediated potentiation of aFGF-induced mitogenesis did not take effect until after the first 24 hours of culture, peaking at the end of the second day. This suggests that, initially, while there is sufficient aFGF to form productive complexes between FGFRs and endogenous HSPGs, heparin cannot enhance receptor activation. As the aFGF is internalized (or degraded), the proportion of unsuccessful complexes increases, due to aFGF's low affinity for cell surface HSPGs (see chapter 3). The presence of heparin enables lower concentrations of aFGF to be utilized more efficiently by increasing the number of productive ternary complexes formed and thereby enabling receptor activation to occur.



#### 4.4 Summary

Recently it was reported (Belford et al., 1992) that carboxyl-reduced heparin (CR-heparin), despite binding aFGF as effectively as native heparin, was much less potent at augmenting aFGF-induced mitogenesis. This chapter describes experiments which examined this phenomenon in more detail in the hope that it would shed light on the mechanism by which heparin potentiates aFGF activity.

Initial studies confirmed that heparin with 60% of its carboxyl groups reduced, although binding aFGF with the same affinity as native heparin ( $K_d$   $35 \pm 5$  nM), was a poor potentiator of aFGF induced mitogenic activity. Proteolysis protection experiments also revealed that CR-heparin was as effective as native heparin at protecting aFGF from proteolytic degradation. In contrast, CR-heparin was considerably less effective than native heparin at enhancing the binding of aFGF to either the FGFR on 3T3 cells or to a soluble form of recombinant murine FGFR1. Furthermore, CR-heparin only bound to a subset (approx. 1/3) of heparin receptors on 3T3 cells and bound weakly to a heparin binding site on FGFR1. Since CR-heparin protected aFGF from proteolysis and bound aFGF with high affinity it appeared unlikely that the inability of CR-heparin to enhance aFGF binding to the FGFR was due to its failure to induce a conformational change in aFGF essential for FGFR binding. In contrast, the reduced ability of CR-heparin to interact with heparin binding sites suggests that it is much less efficient than heparin at facilitating the formation of a ternary complex between aFGF and the FGFR. Such a ternary complex would require a heparin-FGF complex to crosslink the FGFR with a heparin binding site either on the FGFR itself or on adjacent molecules on the cell surface.

Additional studies demonstrated that CR-heparin interacted with bFGF with a binding affinity comparable to native heparin. Nevertheless, unlike native



heparin, CR-heparin was completely unable to restore binding of bFGF to soluble recombinant FGFR1. However, CR-heparin was quite an effective inhibitor of native heparin-induced binding of bFGF to FGFR1. Such results were not obtained with aFGF. These observations suggest that acidic and basic FGF have different requirements for binding to FGFR1 and a model has been proposed to explain these differences.

## Chapter 5

### Histidine-Rich Glycoprotein and Platelet Factor 4 Mask Heparan Sulfate Proteoglycans Recognized by Acidic and Basic Fibroblast Growth Factor

#### 5.1. Introduction

In addition to the heparin-binding growth factors, there are three other well characterized heparin-binding proteins present in plasma. These are histidine-rich glycoprotein (HRG), platelet factor 4 (PF4) and antithrombin III (ATIII). Since recent studies have shown that FGFs need to interact with cell surface HSPGs in order to bind to and activate the FGFR (Yayon et al.,1991), I have attempted in this chapter to investigate whether HRG, PF4 or ATIII are able to act as modulators of FGF activity *in vivo*. If able to compete with FGFs for binding to cell-surface HSPGs, it is proposed that these molecules may be able to maintain an equilibrium in which FGFs are only able to access their receptors when their activity is specifically required.

Histidine-rich glycoprotein, with a molecular weight of 75kDa, is a major heparin-binding protein present in plasma (Haupt & Heimburger 1972; Lijnen et al.,1983b), being present in mammalian plasma at concentrations as high as 100ug/ml (Lijnen et al.,1983a). As is suggested by its name, HRG contains a remarkably high content of histidine and proline residues, these residues comprising approximately 20% of its amino acids (Heimburger et al 1972; Lijnen et al.,1980; Koide et al.,1985). Although little is known about HRG, data suggests that it binds to unique HS sequences and that its heparin/HS binding domain probably lies within the histidine-rich region (Burch et al.,1987). It also binds to plasminogen (Lijnen et al.,1980; Ichinose et al.,1984), fibrinogen (Ichinose et al.,1984) and thrombospondin (Leung.,1986). Some of the functions that have been assigned to HRG to date are the neutralization of heparin's anticoagulant activity in plasma (Lijnen et al.,1983b), the



neutralization of heparin's ability to inhibit smooth muscle cell proliferation (Hajjar et al.,1987), inhibition of cell adhesion (Rylatt et al.,1981; Silverstein et al.,1985), immunosuppression (Shatsky et al.,1989), retardation of fibrinolysis by plasmin, and by binding to fibrinogen, retardation of the conversion of fibrinogen to fibrin by thrombin (Leung 1986).

Platelet factor 4 exists as a high molecular weight complex of a tetramer of PF4 polypeptide and chondroitin sulfate (Barber et al.,1972; Moore et al.,1975) in the  $\alpha$  granules of platelets. It also has a high affinity for heparin (Maione et al.,1990), eluting from heparin-Sepharose with 1.5M NaCl (Niewiarowsky et al.,1976). Although normally found in human plasma at concentrations as low as 10ng/ml, concentrations as high as 25ug/ml are thought to be obtained at sites of vascular injury where platelets aggregate and the  $\alpha$ -granules are released (Files et al.,1981). The physiological significance of PF4 is uncertain although it does reverse immunosuppression (Katz et al.,1986), has chemotactic activity for neutrophils, monocytes, and fibroblasts (Deuel et al.,1981; Senior et al 1983) inhibits bone resorption (Horton et al.,1980) and inhibits angiogenesis (Taylor & Folkman 1982). In addition to its anti-angiogenic activity, Sharpe et al.,(1990) have shown that PF4 specifically inhibits endothelial cell proliferation and migration *in vitro* and the active part of the molecule has been identified as the heparin-binding region. However, the systemic administration of hrPF4 has not been shown to inhibit tumour growth possibly due to its rapid inactivation or clearance (Folkman & Brem 1992). The demonstration by Maione et al.,(1990) that inhibition of HUVE cell proliferation *in vitro* by rhPF4 could be reversed by addition of fresh medium containing growth factor and heparin, suggested that PF4-induced inhibition was due to suppression of growth factor activity. However, the observation that PF4 did not inhibit bFGF-induced proliferation of human dermal fibroblasts or keratinocytes led them to conclude that rhPF4 inhibition of HUVE cell proliferation was not due to binding and sequestering of soluble bFGF.



Antithrombin III, present in human plasma at concentrations of 150ug/ml (Murano et al., 1980), is the major inhibitor of the activated serine proteases of the blood coagulation cascade. ATIII binds to the serine proteases and forms a stable and inactive complex with them (Rosenberg & Damus 1973; Owen 1975). The binding of heparin to ATIII greatly facilitates the inactivation of the enzymes involved in the coagulation process (Bjork & Nordenman 1976; Kowalski & Finlay 1979; Holmer et al., 1979). Heparin is thought to induce a conformational change in either ATIII or the serine protease facilitating the formation of the inhibitor-protease complex (Rosenberg & Damus 1973; Li et al., 1976). While it is not known to which heparin/HS sequences HRG and PF4 bind, ATIII is known to bind specifically to a particular pentasaccharide in heparin and HS of which the 3-O-sulfate group of the internal glucosamine residue is essential for high affinity binding (Petitou et al., 1988).

In the following section, I have outlined studies conducted to determine whether chicken and human HRG, human ATIII and human PF4 interact with the same HSPGs on the 3T3 cell surface and in the ECM as do acidic and basic FGF. The study also compares the relative binding affinities of these heparin-binding molecules for HSPGs and compares their abilities to inhibit the binding of  $^{125}\text{I}$ -aFGF and  $^{125}\text{I}$ -bFGF to the 3T3 cell surface and to the ECM.

## **5.2. Results**

### **5.2.1. Binding of HRGs to Cell Surface HSPGs**

In chapter 3 an assay was developed for detecting the binding of acidic and basic FGF to cell surface HSPGs on BALB/c 3T3 cells. The assay entailed immobilizing the growth factors in PVC plastic wells and quantifying cell adhesion to the growth factors using the vital stain Rose Bengal. In the case of HRG, preparations from human (hHRG) and chicken (cHRG) plasma were



prepared by phosphocellulose ion exchange chromatography (Rylatt et al., 1981) and used in subsequent experiments. The purity of the two HRG preparations was demonstrated by SDS-PAGE, in each case a single Coomassie Blue stained band being detected (Fig. 5.1). Human HRG has a molecular weight of approximately 75kDa whereas chicken HRG is a somewhat larger molecule with a molecular weight of approximately 135kDa.

In initial experiments the optimum concentration of chicken and human HRG for coating PVC plates for use in the adhesion assay was determined (Fig. 5.2). It was found that both HRGs could mediate BALB/c 3T3 cell adhesion when immobilized to plastic, although lower coating concentrations of cHRG than hHRG were required for maximum binding. In subsequent assays, cHRG was used at 10ug/ml and hHRG at 5ug/ml to coat the plates.

An interesting feature of this adhesion assay was that plastic immobilized HRG is able to act as a spreading factor for BALB/c 3T3 cells in the absence of fetal calf serum. Figure 5.3 shows that after two hours incubation at 37°C, cells added to an HRG-coated dish had attached quite firmly to the HRG substrate and had changed from a rounded to a more spread out morphology. In contrast, cells seeded on an uncoated dish remained rounded and showed no inclination to attach to the plastic surface.

The next series of experiments examined whether binding of BALB/c 3T3 cells to immobilized HRG was heparin inhibitable as was shown to be the case for immobilized acidic and basic FGF (chapter 3). Eleven GAGs were tested at a single concentration (100ug/ml) for their ability to inhibit the binding of BALB/c 3T3 cells to either chicken or human HRG-coated wells. As was found for the FGFs, only heparin, fast moving HS and bovine intestinal HS were able to inhibit HRG binding. Figure 5.4 depicts the complete inhibition curves for heparin, fast moving HS and bovine intestinal HS. Heparin was the most

FIGURE 5.1: SDS-PAGE analysis of preparations of chicken and human HRG. Samples (10ug) were run on a 10% gel and stained with Coomassie Blue. Molecular weight standards are marked in kDa.



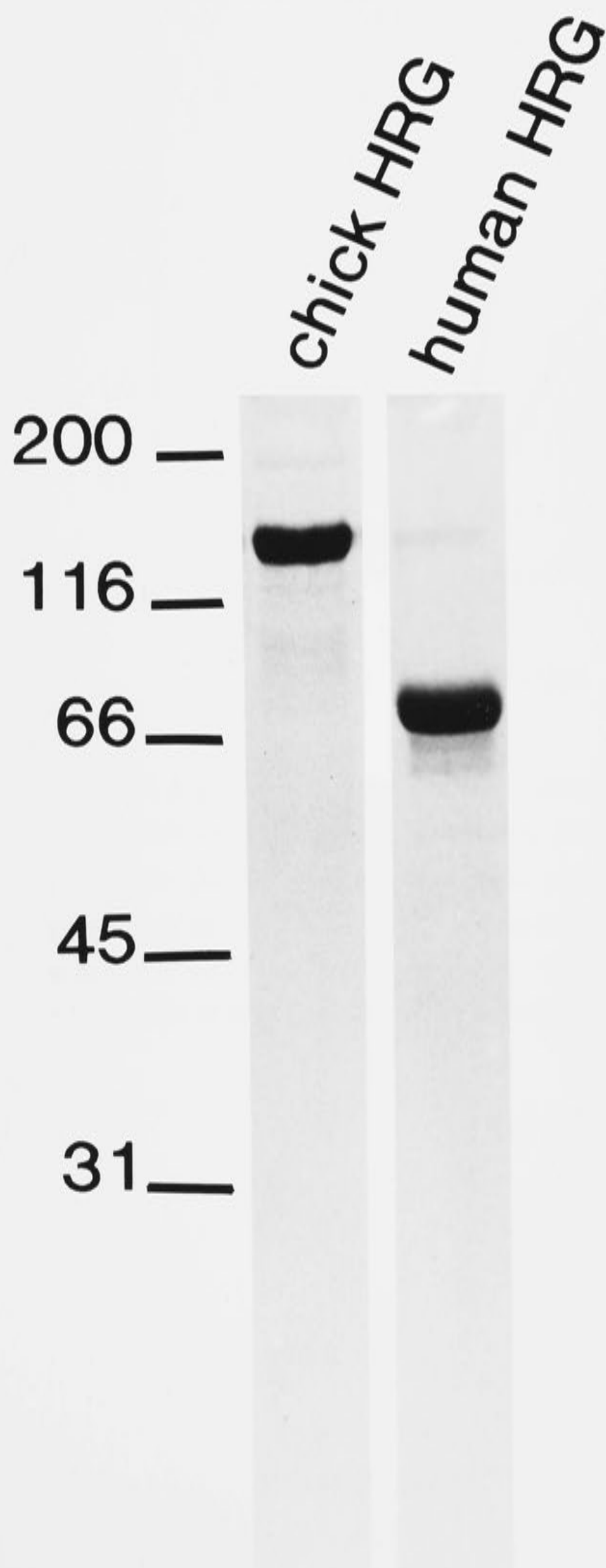


FIGURE 5.3: Ability of cHRG to promote the attachment and spreading of BALB/c 3T3 cells. Bacteriological petri dishes were coated with PBS (A) or with cHRG (10ug/ml) in PBS (B) and non specific binding sites blocked with Hanks BSS/0.1% BSA pH 7.0 before the addition of 10ml of a BALB/c 3T3 cell suspension ( $0.6 \times 10^5$  cells/ml) to each dish. The cells were left to adhere to the dishes for 2 hours at 37°C before being examined under a light microscope.



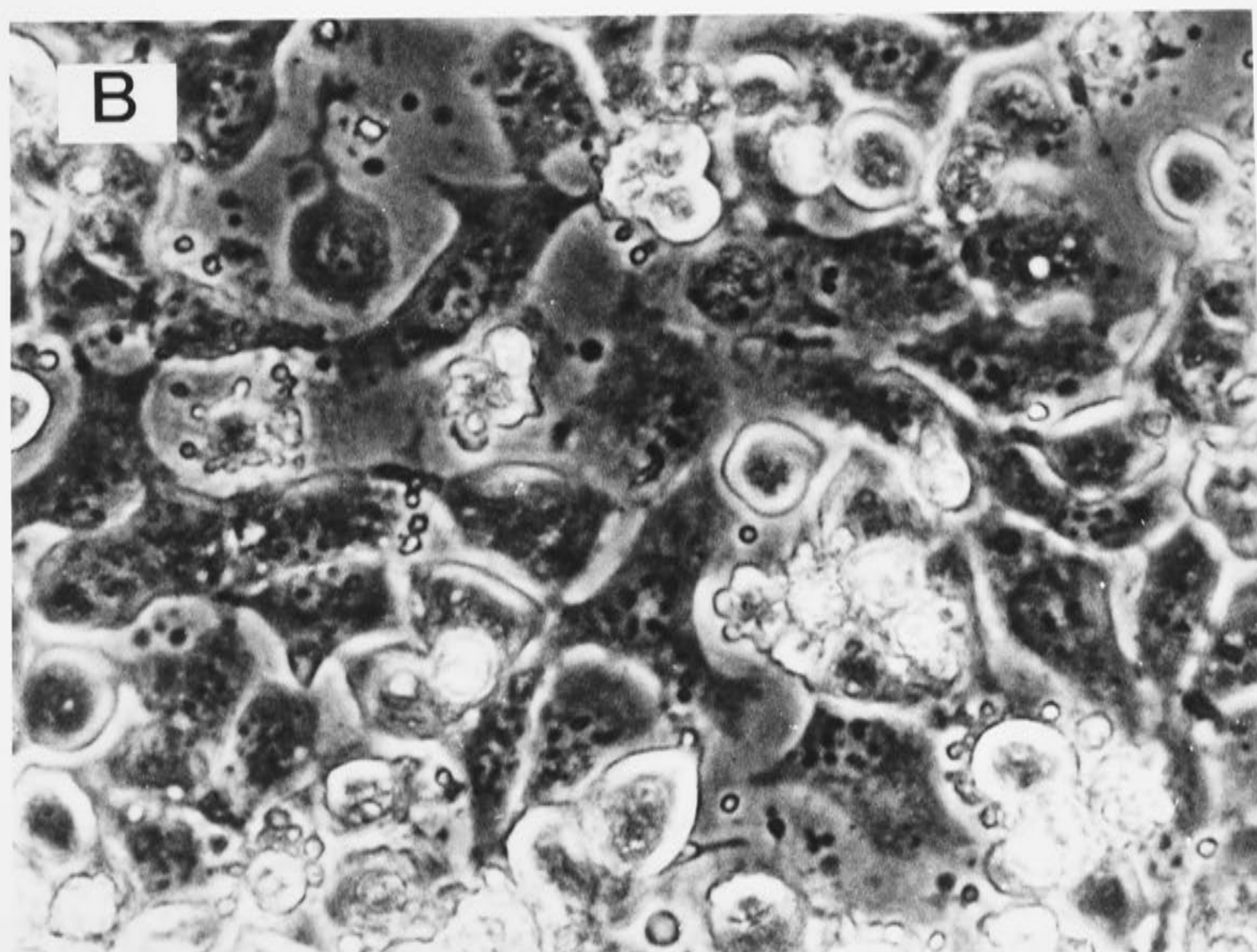
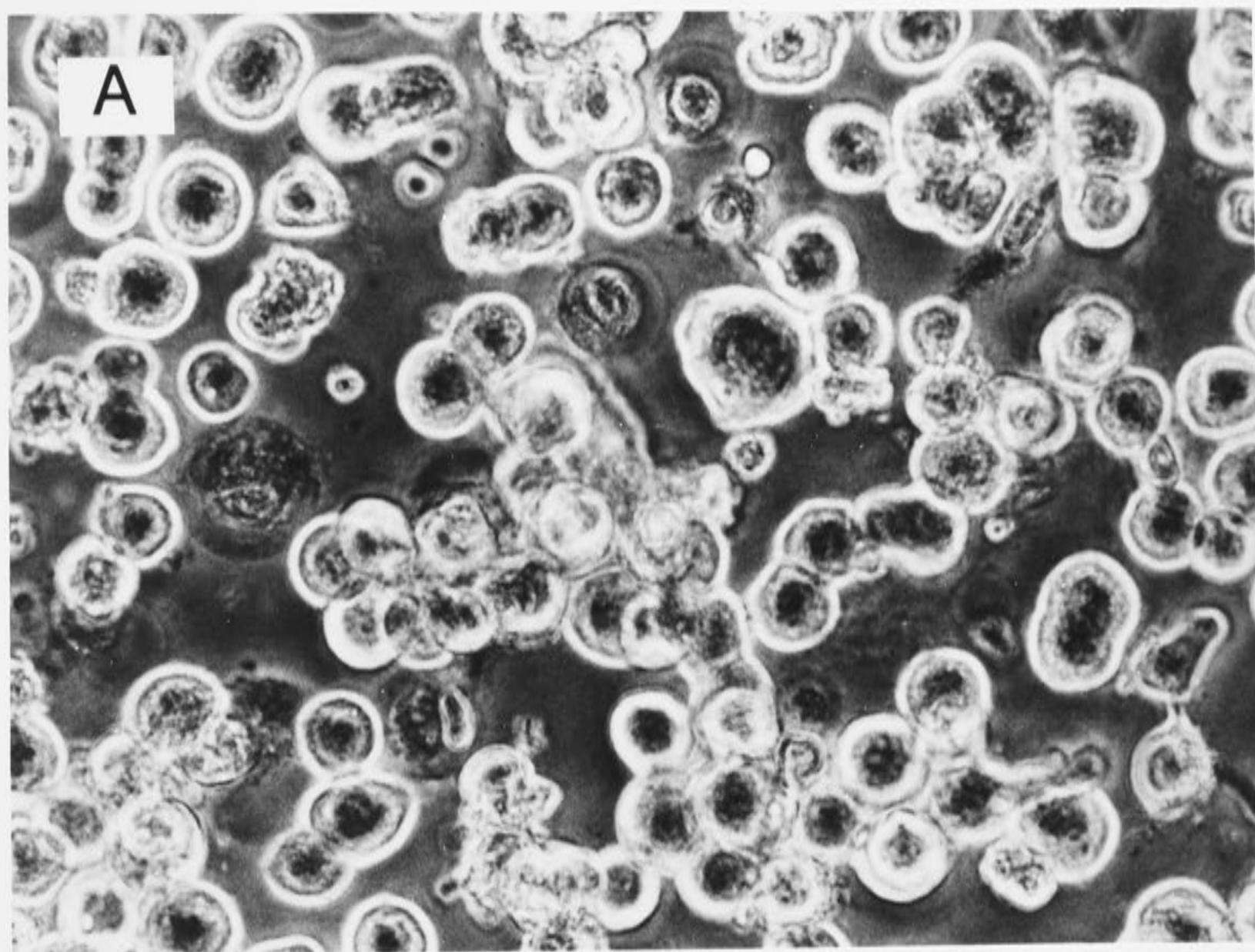
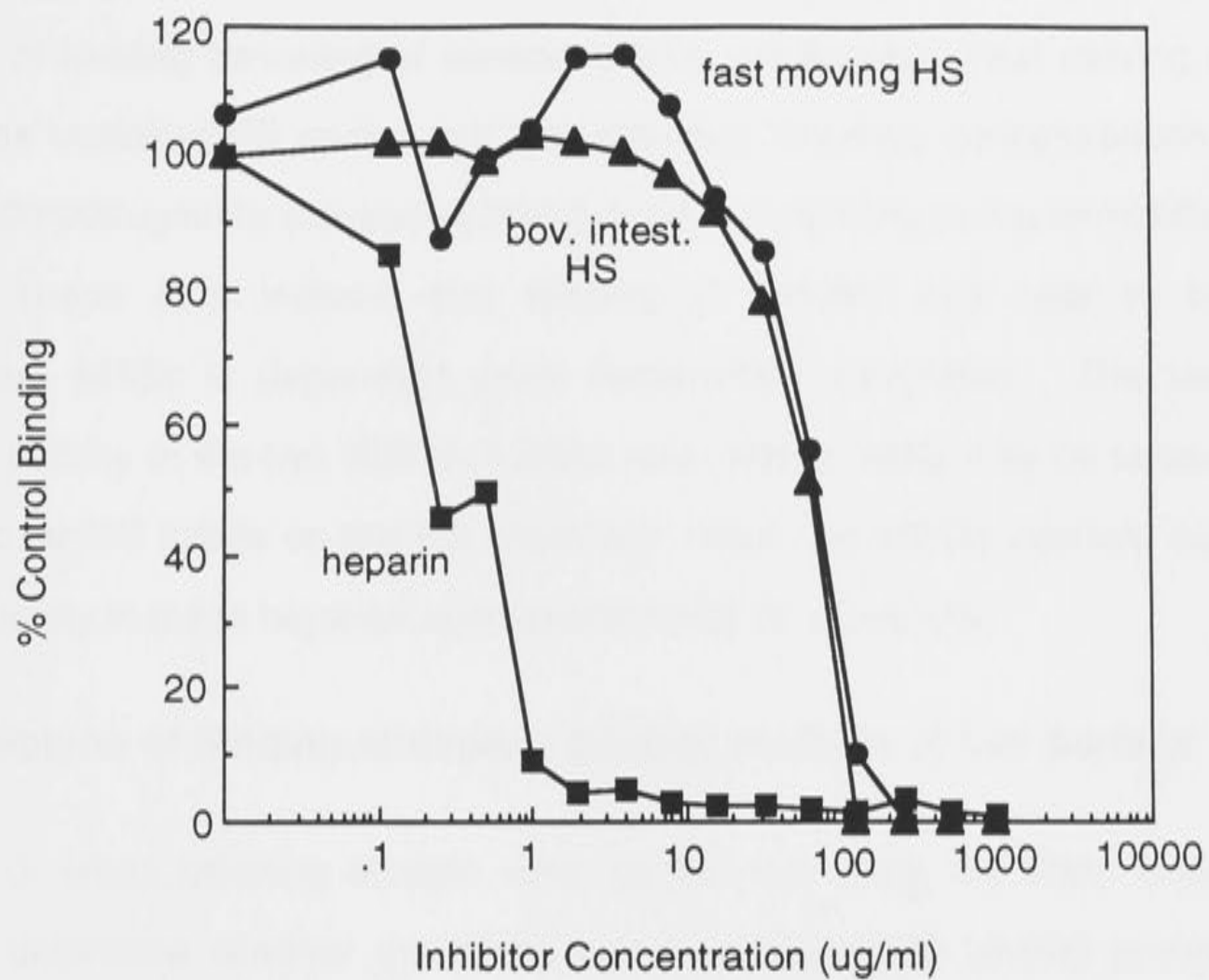




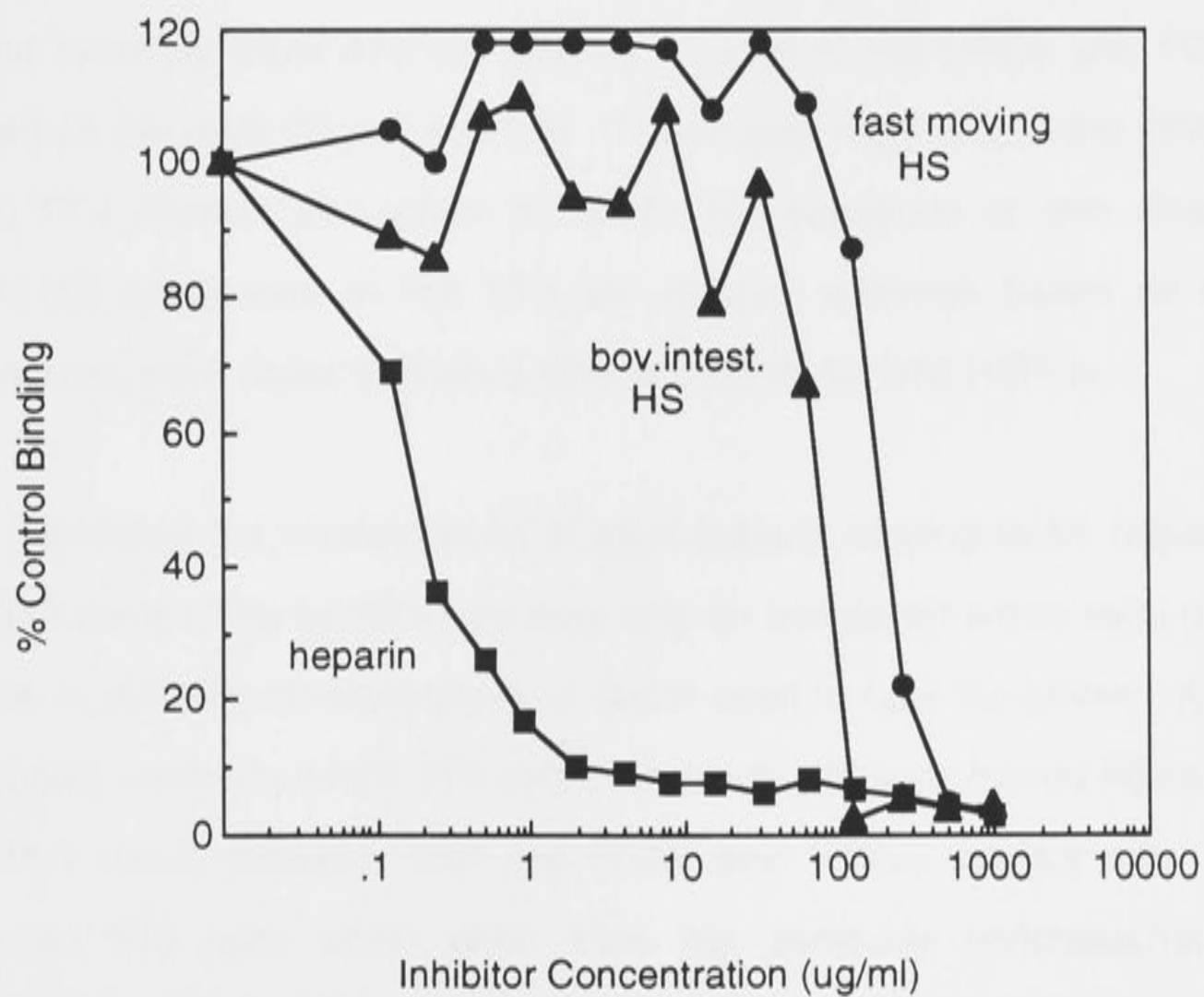
FIGURE 5.4: Comparison of the ability of heparin (■), fast moving heparan sulfate (●) and bovine intestinal heparan sulfate (▲) to inhibit the binding of BALB/c 3T3 cells to immobilized (A) cHRG (10ug/ml) and to (B) hHRG (5ug/ml) . Heparin and heparan sulfates were incubated with immobilized HRG for 1 hour on ice before adding cells ( $2.5 \times 10^5$  cells/well) for 1 hour at 37°C, 100% binding representing that occurring in the absence of inhibitor. Values represent the means of triplicate treatments. Standard errors of the mean were < 10%.



A)



B)



potent competitor for both cHRG and hHRG binding, with virtually complete inhibition of binding occurring at concentrations  $> 0.8\mu\text{g/ml}$ . Fast moving HS and bovine intestinal HS were much less effective, requiring concentrations as high as 100-300 $\mu\text{g/ml}$  to completely inhibit cells from binding to the immobilized HRGs. These data indicate that binding of BALB/c 3T3 cells to both immobilized HRGs is dependent upon heparin/HS recognition. The lower inhibitory activity of the two HSs for human and chicken HRG may be because the particular HS motifs on the HS chain with which the HRGs interact, occur less frequently in these heparan sulfates than they do in heparin.

#### **5.2.2. Inhibition of Binding of Heparin Binding Proteins to Cell Surface HSPGs**

A series of cross blocking studies were carried out using the plate binding assay to determine whether the FGFs and the heparin/HS binding proteins, HRG, ATIII and PF4 bind to similar or different HS structures on the surface of BALB/c 3T3 cells. Chicken and human HRG, acidic and basic FGF, and PF4 were found to totally block 3T3 cell binding to each of the HRGs and FGFs immobilized on the plate (Figs 5.5 & 5.6). These data suggest that the HRGs, FGFs and PF4 interact with either the same HS sequence or with closely associated HS sequences on the 3T3 cell surface, although based on the inhibition curves, each factor exhibits a different affinity for 3T3 HSPGs.

Table 5.1 compares the concentration of each heparin binding factor required to inhibit 3T3 cell binding by 50% and may only be compared within each data column due to different concentrations of factor used to coat the plates. ATIII was completely unable to inhibit 3T3 cells from binding to immobilized FGFs, or HRGs. This result indicates that the FGFs and HRGs interact with HS structures on 3T3 cells which differ from the particular pentasaccharide sequence in heparin and HS with which ATIII is known to bind. Based on the inhibition data presented in Table 5.1 the overall hierarchy of inhibition for the five inhibitory factors was  $\text{PF4} \geq \text{bFGF} > \text{aFGF} = \text{cHRG} > \text{hHRG}$ . This



Table 5.1

Ability Of Different Heparin Binding Molecules To Inhibit Binding of FGFs and HRGs to 3T3 Cells

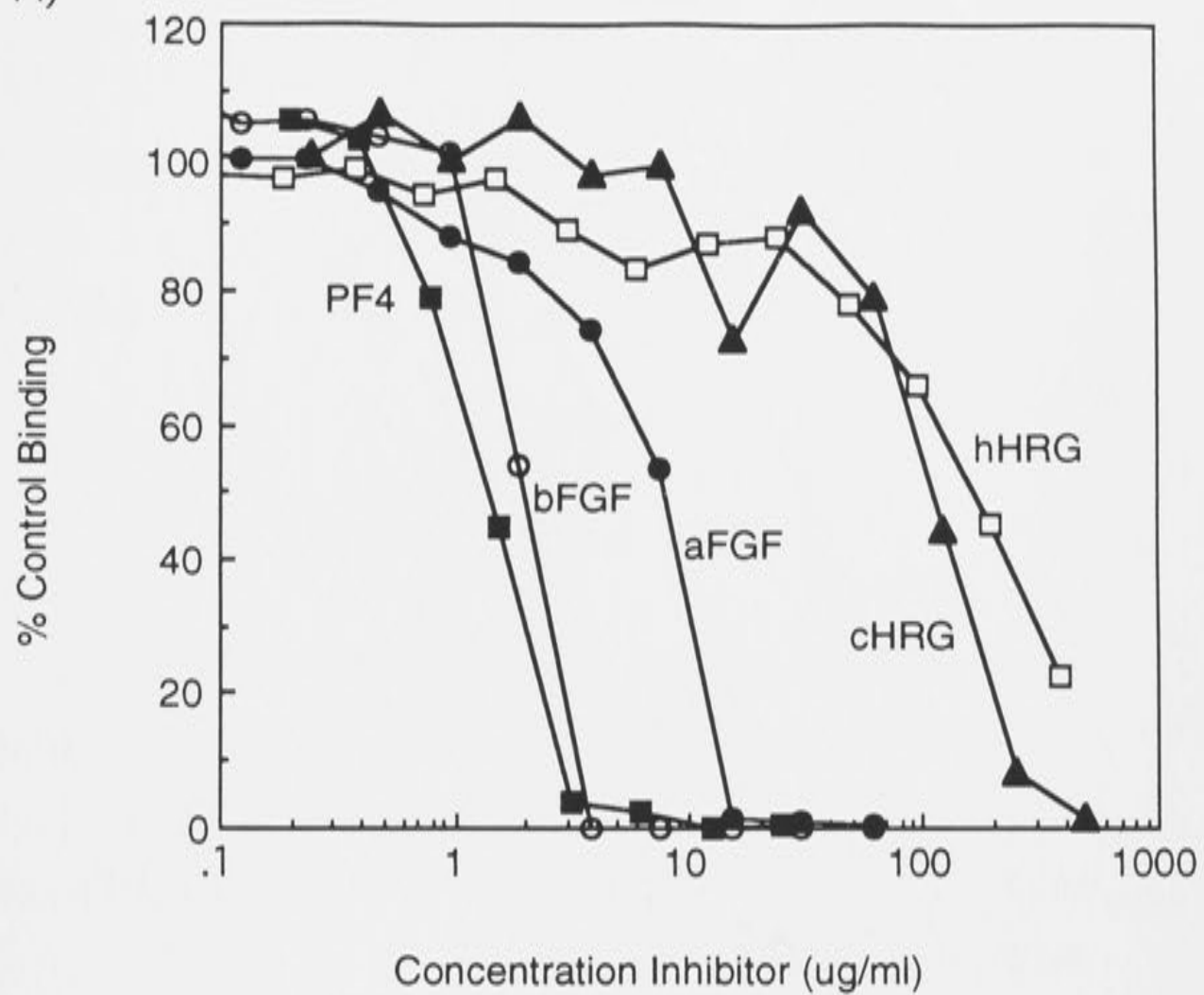
Inhibitor	Molecule Bound to Plate			
	bFGF	aFGF	cHRG	hHRG
PF4	64 <sup>a</sup>	42	33	21
bFGF	34	200	114	200
aFGF	125	1875	500	625
cHRG	148	815	940	407
hHRG	2667	5333	2400	2000
ATIII	>16000	>16000	>16000	>16000

<sup>a</sup> Values represent the concentration (nM) required to inhibit BALB/c 3T3 cell binding by 50%, based on molecular weights of inhibitors being 31.2kDa for PF4, 17.5kDa for bFGF, 16kDa for aFGF, 135kDa for cHRG, 75kDa for hHRG and 62.3 kDa for ATIII.

FIGURE 5.5: Comparison of the ability of soluble PF4 (■), bFGF (○), aFGF (●), cHRG (▲) and hHRG (□) to inhibit the binding of BALB/c 3T3 cells to immobilized (A) cHRG (10ug/ml) and to (B) hHRG (5ug/ml). Cells and inhibitors were incubated together for 2 hours on ice before being added to HRG-coated wells for a further 1 hour incubation at 37°C, 100% binding representing that occurring in the absence of inhibitor. Values represent the mean of triplicate treatments. Standard errors of means were < 20%.



A)



B)

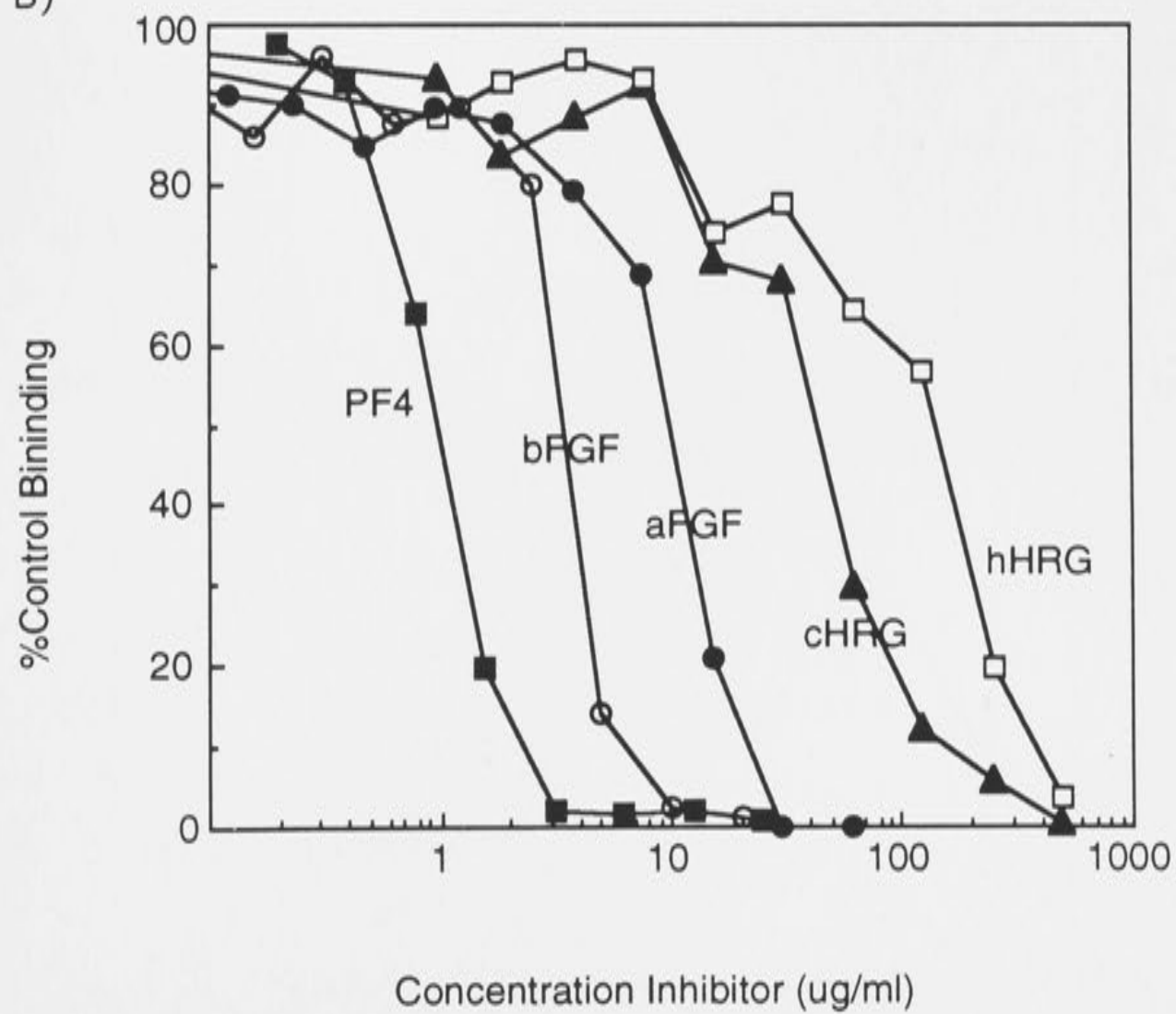
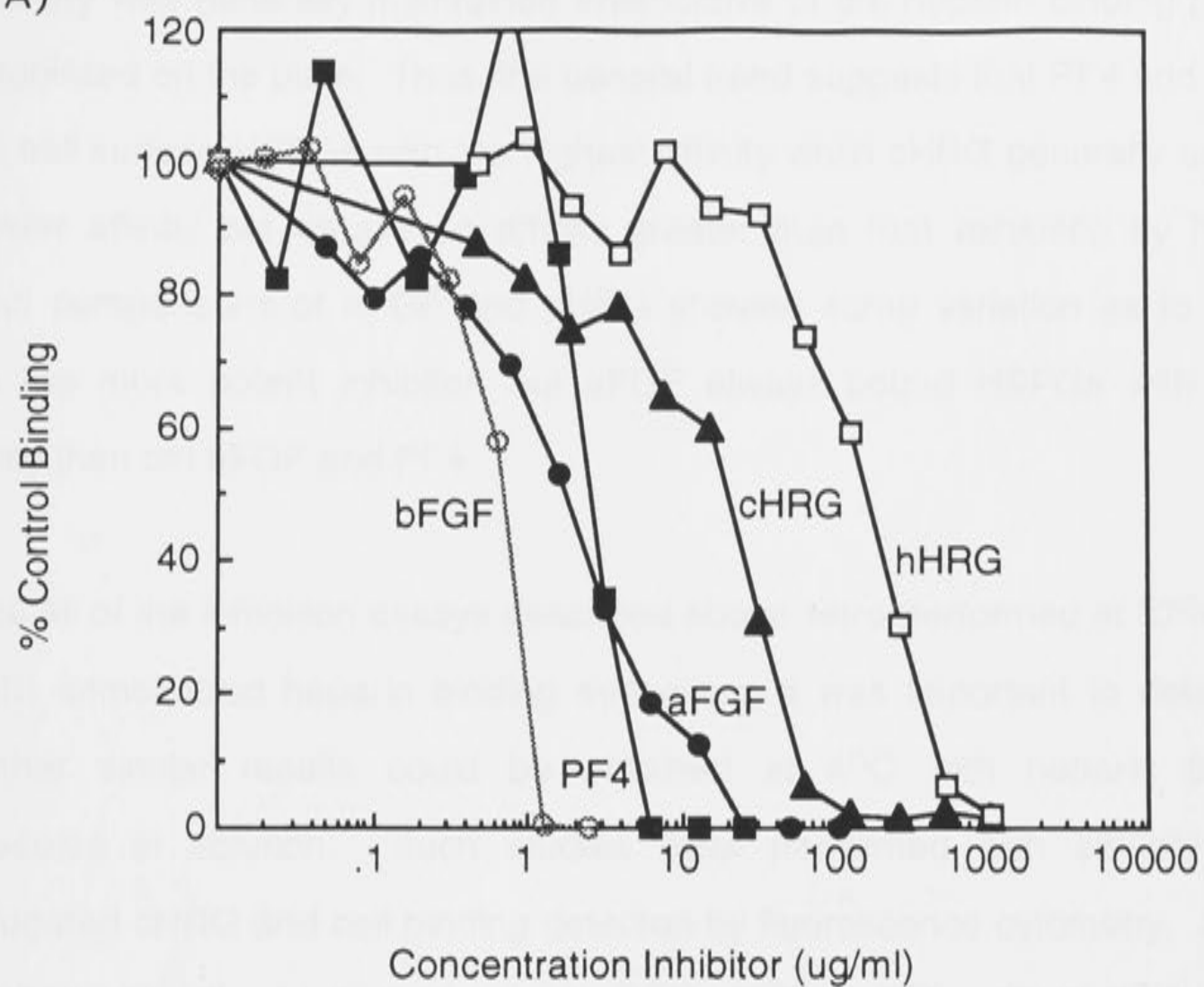


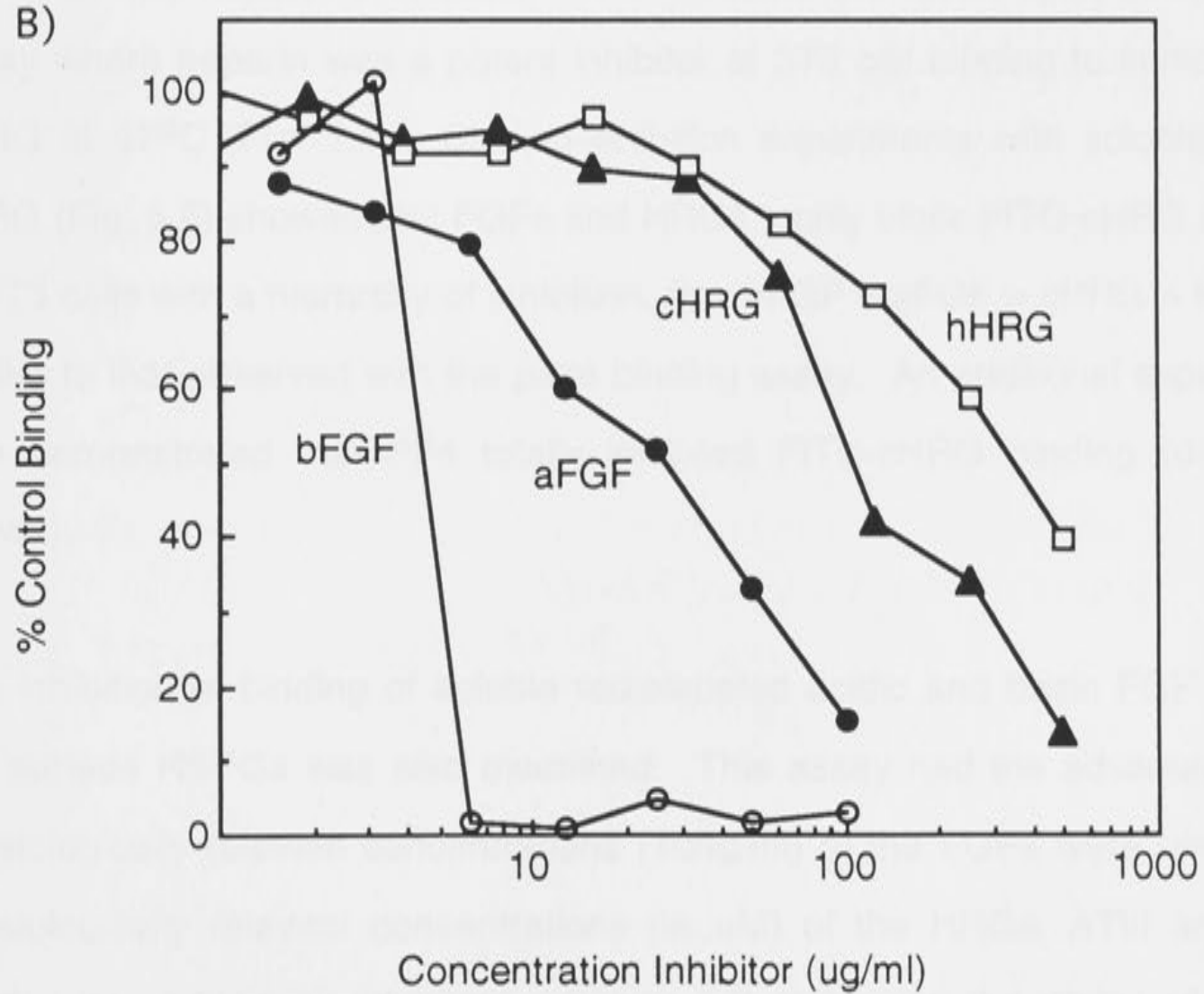
FIGURE 5.6: Comparison of the ability of soluble bFGF (○), PF4 (■), aFGF (●), cHRG (▲) and hHRG (□) to inhibit the binding of BALB/c 3T3 cells to immobilized (A) bFGF (0.313ug/ml) and to (B) aFGF (2.5ug/ml). Assay conditions and data presentation as described in legend for Figure 5.5.



A)



B)



hierarchy was generally maintained irrespective of the heparin binding protein immobilized on the plate. Thus, the general trend suggests that PF4 and bFGF bind cell surface HSPGs with the highest affinity while cHRG generally exhibits a lower affinity but always an affinity greater than that exhibited by hHRG. Direct comparisons of aFGF and cHRG showed some variation as to which was the more potent inhibitor, but aFGF always bound HSPGs with lower affinity than did bFGF and PF4.

Since all of the inhibition assays described above were performed at 37°C with plastic immobilized heparin binding molecules, it was important to determine whether similar results could be obtained at 4°C with heparin binding molecules in solution. Such studies were performed with soluble FITC conjugated cHRG and cell binding detected by fluorescence cytometry. Figure 5.7 shows that the binding of soluble FITC-cHRG to 3T3 cells at 4°C is both saturable and heparin inhibitable. This confirms the result seen in the plate assay where heparin was a potent inhibitor of 3T3 cell binding to immobilized cHRG at 37°C (Fig. 5.4). Binding-inhibition experiments with soluble FITC-cHRG (Fig. 5.8) showed that FGFs and HRGs totally block FITC-cHRG binding to 3T3 cells with a hierarchy of inhibition, (ie., bFGF > aFGF = cHRG > hHRG), similar to that observed with the plate binding assay. An additional experiment also demonstrated that PF4 totally inhibited FITC-cHRG binding (data not shown).

The inhibition of binding of soluble radiolabeled acidic and basic FGF to 3T3 cell surface HSPGs was also examined. This assay had the advantage that physiologically relevant concentrations (10ng/ml) of the FGFs were used with physiologically relevant concentrations (ie., uM) of the HRGs, ATIII and PF4 which were added as inhibitors. Figure 5.9 compares the ability of bFGF, aFGF, cHRG, hHRG and ATIII to inhibit the binding of 10ng/ml of <sup>125</sup>I-bFGF to the 3T3 cell surface. The results are in agreement with those obtained with the



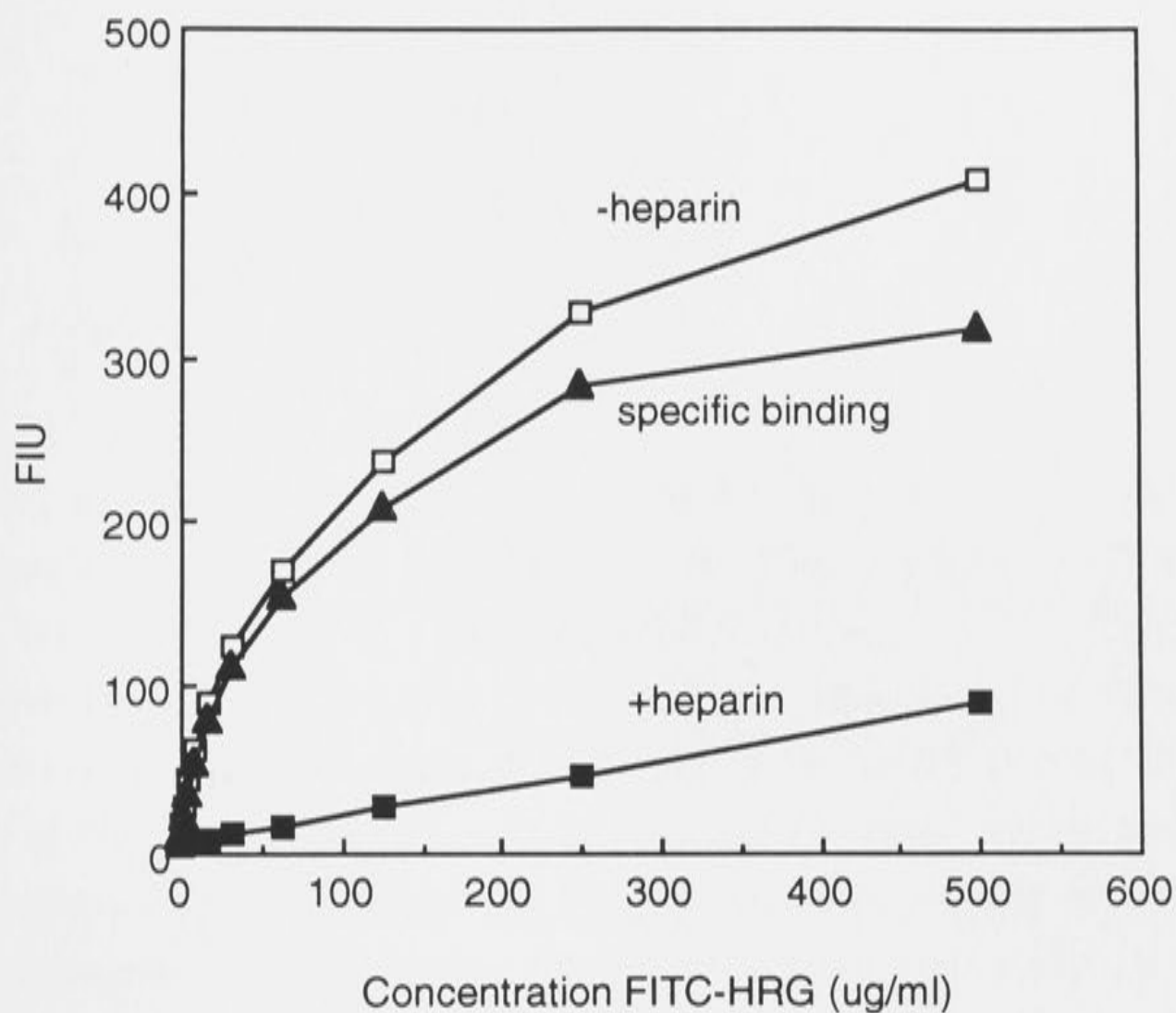


FIGURE 5.7: Ability of FITC-cHRG to bind to BALB/c 3T3 cells in the presence and absence of heparin (200 $\mu\text{g/ml}$ ). FITC-cHRG and heparin were incubated together for 30min on ice before being added to cells and incubated for a further 30min on ice. After washing, the FITC-cHRG remaining bound to the cells in the presence and absence of heparin was determined by a FACSscan. Amount of FITC-cHRG bound to cells is given in fluorescence intensity units (FIU).

FIGURE 5.8: Ability of different concentrations of (A) cHRG ( ▲ ) and hHRG ( □ ) and (B) aFGF ( ● ) and bFGF ( ○ ) to inhibit the binding of FITC-cHRG (50ug/ml) to BALB/c 3T3 cells. cHRG, hHRG, aFGF or bFGF were incubated with BALB/c 3T3 cells for 30min on ice before FITC-cHRG was added to each sample and incubated for a further 1 hour on ice. After washing, the FITC-cHRG remaining bound to the cells in the presence and absence of inhibitor was determined by a FACScan. The amount of FITC-cHRG bound to cells is expressed as a percent of control binding, ie., that occurring in the absence of inhibitor.



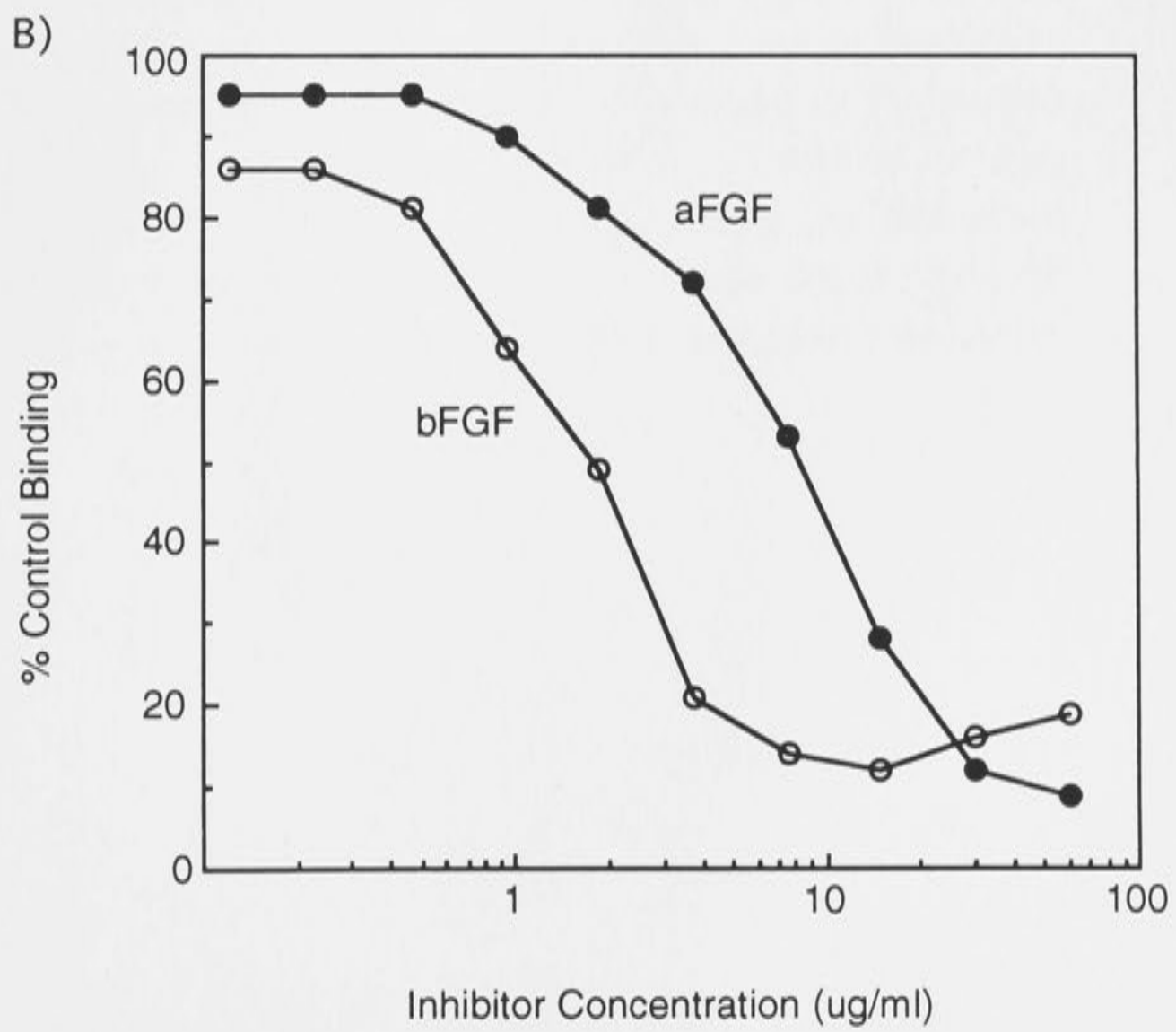
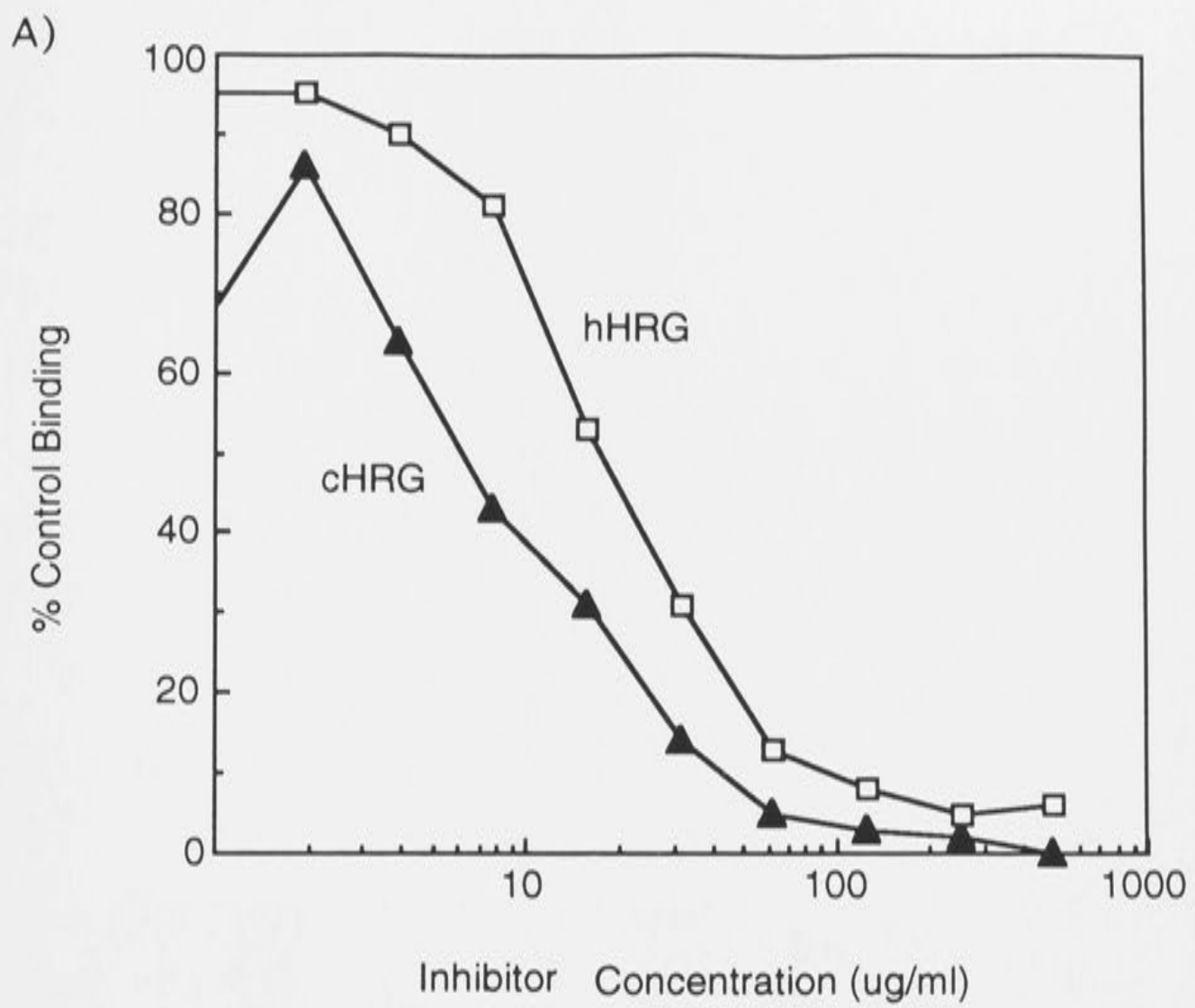
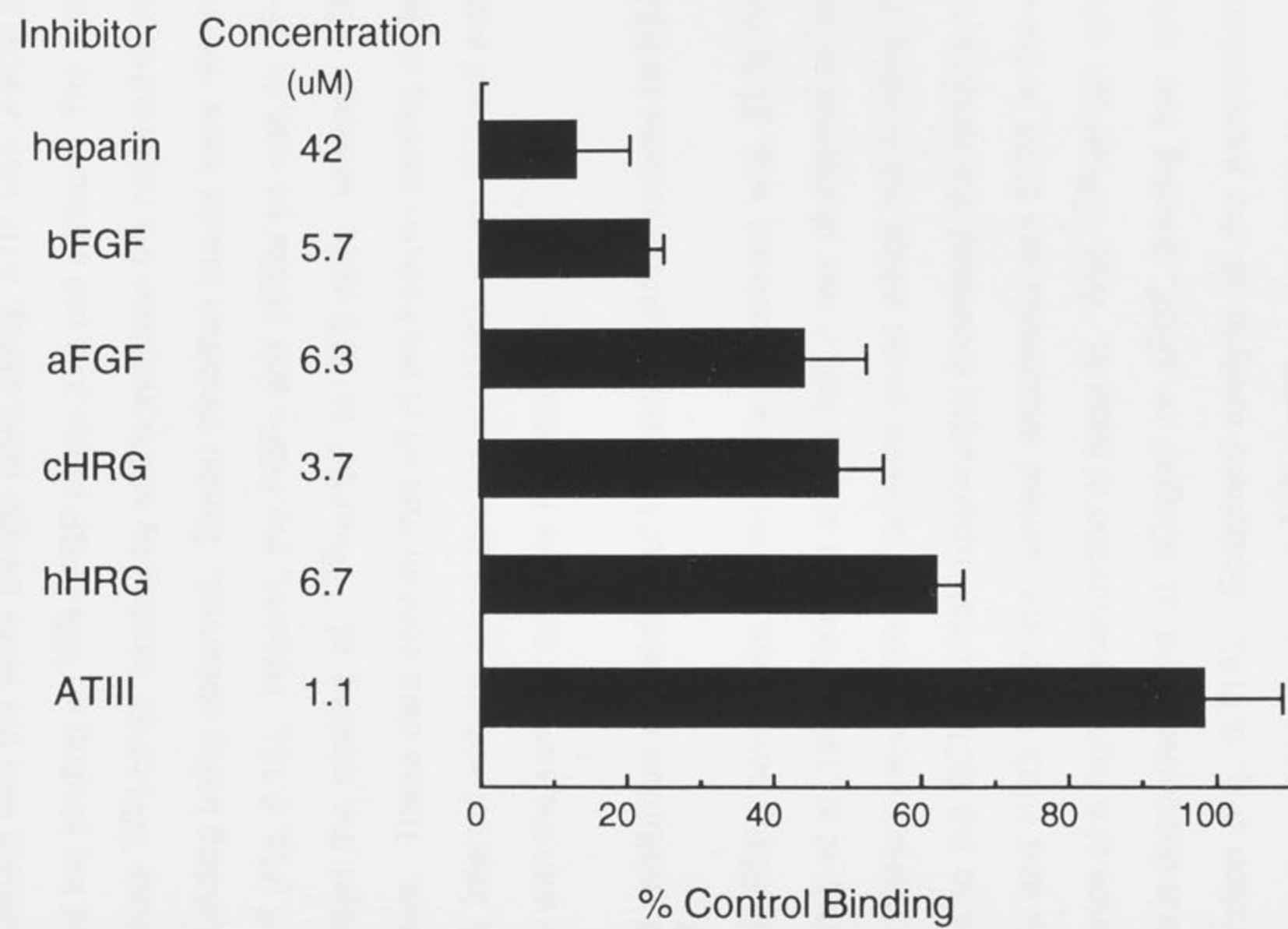


FIGURE 5.9: Ability of heparin, bFGF, aFGF, cHRG, hHRG and ATIII to inhibit the binding of  $^{125}\text{I}$ -bFGF (10ng/ml; 0.57nM) to BALB/c 3T3 cells. Cells were incubated with the inhibitors for 1 hour on ice before the addition of  $^{125}\text{I}$ -bFGF and a further 2 hours incubation on ice. After washing away unbound  $^{125}\text{I}$ -bFGF, the amount of cell-associated radioactivity was determined by a gamma counter.  $^{125}\text{I}$ -bFGF binding to the cell surface is presented as percent control binding, ie.,  $^{125}\text{I}$ -bFGF binding occurring in the absence of inhibitor. Each value represents the mean of triplicate treatments  $\pm$  SEM.





Rose Bengal plate assays (Table 5.1). Basic FGF was the most potent inhibitor of  $^{125}\text{I}$ -bFGF binding, reducing binding to <25% of that observed in the absence of inhibitor. Acidic FGF, cHRG and hHRG were somewhat less effective inhibitors reducing binding to approximately 40-60% of the control. In agreement with the Rose Bengal plate assay, ATIII was unable to significantly inhibit the binding of  $^{125}\text{I}$ -bFGF to the 3T3 cell surface. The ability of heparin to inhibit  $^{125}\text{I}$ -bFGF binding by >80% indicates the heparin/HS dependence of the binding being observed. Similar inhibition results were obtained with  $^{125}\text{I}$ -aFGF (Fig. 5.10). Heparin, the FGFs and HRGs as well as PF4 significantly inhibited the binding of  $^{125}\text{I}$ -aFGF to 3T3 cells, whereas ATIII was again inactive. These data confirm that all of the heparin binding molecules, except ATIII, interact with the same species of HSPG on the 3T3 cell surface and very likely with the same or adjacent HS sequences.

### 5.2.3. Inhibition of Binding of Heparin Binding Proteins to HSPGs on the ECM

Since FGFs have been shown to be associated with ECM HSPGs *in vivo* (Jeanny et al., 1987; Folkman et al., 1988) it was of interest to assess whether the different heparin binding proteins could inhibit the binding of radiolabeled FGFs to the ECM. Initial experiments examined the binding of radiolabeled FGFs and HRG to bovine corneal endothelial cell ECM in the presence and absence of a high concentration of heparin. With  $^{125}\text{I}$ -aFGF, binding is clearly heparin-dependent and at 50ug/ml of aFGF, binding has still not reached saturation (Fig. 5.11A). Scatchard analysis of the experimental data found heparin-inhibitable binding of  $^{125}\text{I}$ -aFGF to the ECM to have a dissociation constant of  $2.7 \pm 0.5 \mu\text{M}$ . A similar experiment was performed with  $^{125}\text{I}$ -bFGF and its  $K_d$  determined as  $1.7 \pm 0.2 \mu\text{M}$  (results not shown). Figure 5.11B demonstrates that  $^{125}\text{I}$ -cHRG can also bind to the ECM in a heparin-dependent and saturable manner. Its dissociation constant for heparin-inhibitable binding sites was determined as  $146 \pm 37 \text{ nM}$ . At concentrations of  $^{125}\text{I}$ -cHRG above those indicated, binding could not be inhibited by heparin



FIGURE 5.10: Ability of bFGF, heparin, aFGF, PF4, cHRG, hHRG and ATIII to inhibit the binding of  $^{125}\text{I}$ -aFGF (10ng/ml; 0.63nM) to BALB/c 3T3 cells. Assay conditions and data presentation as described in the legend of Figure 5.9.

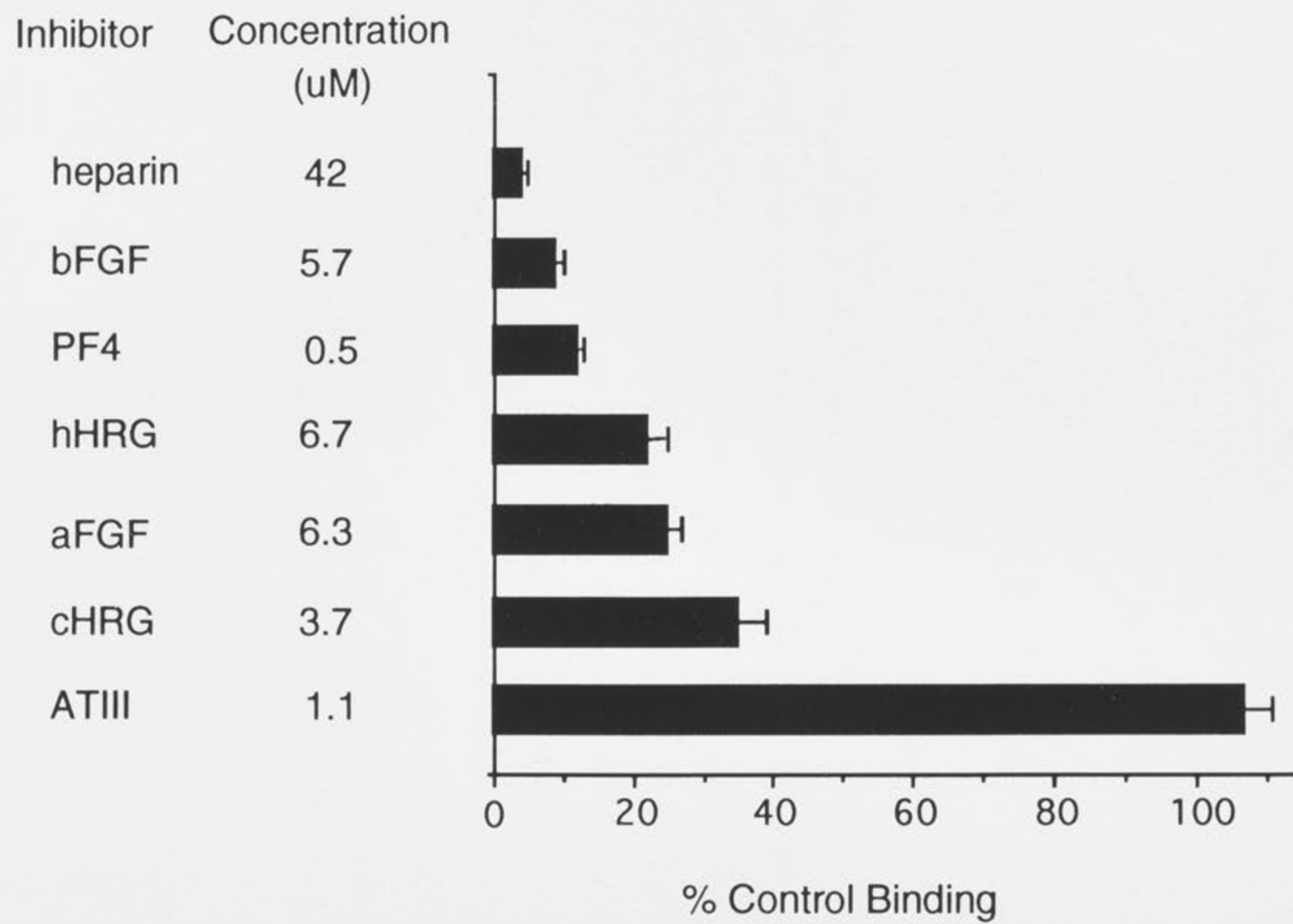
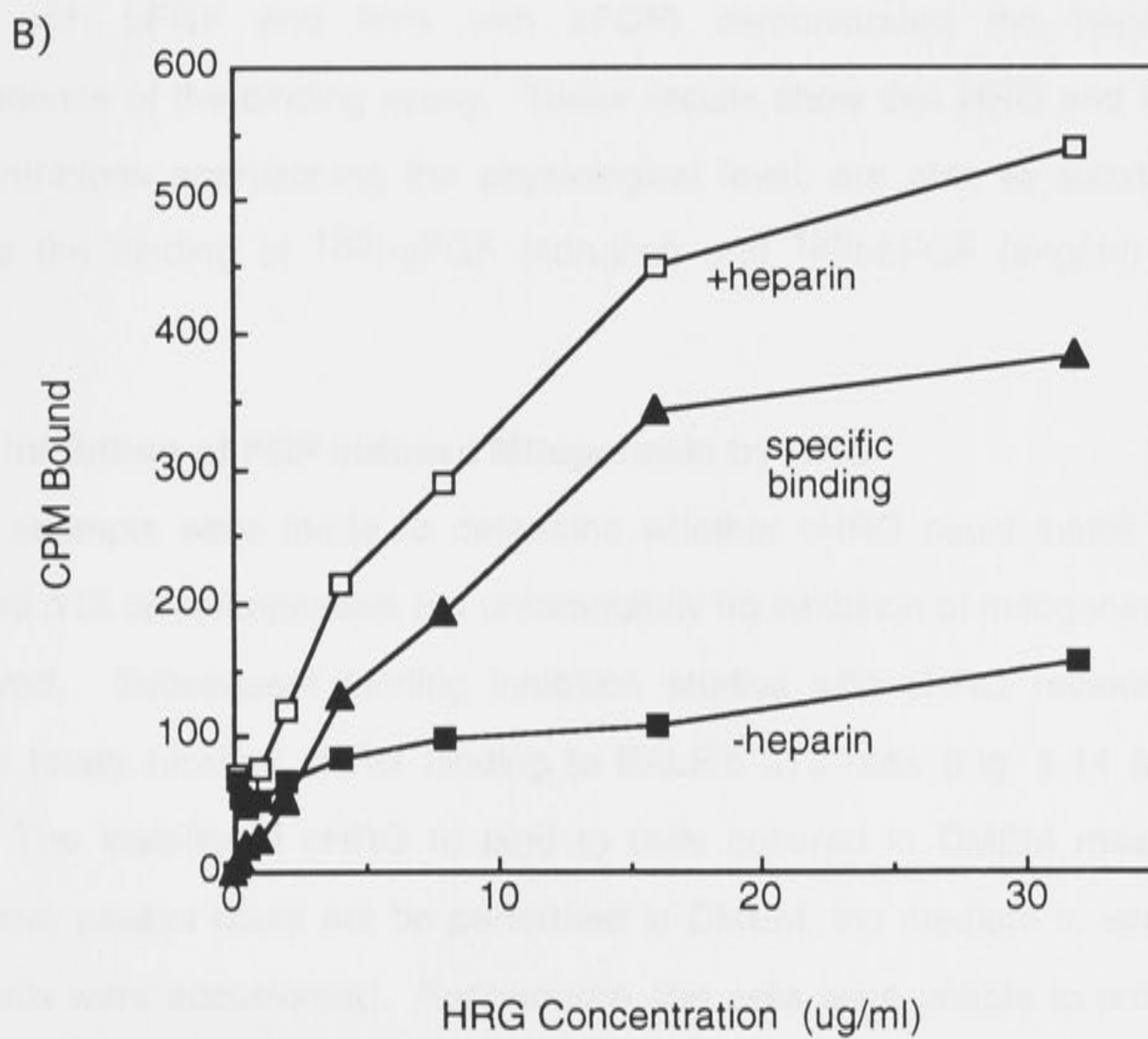
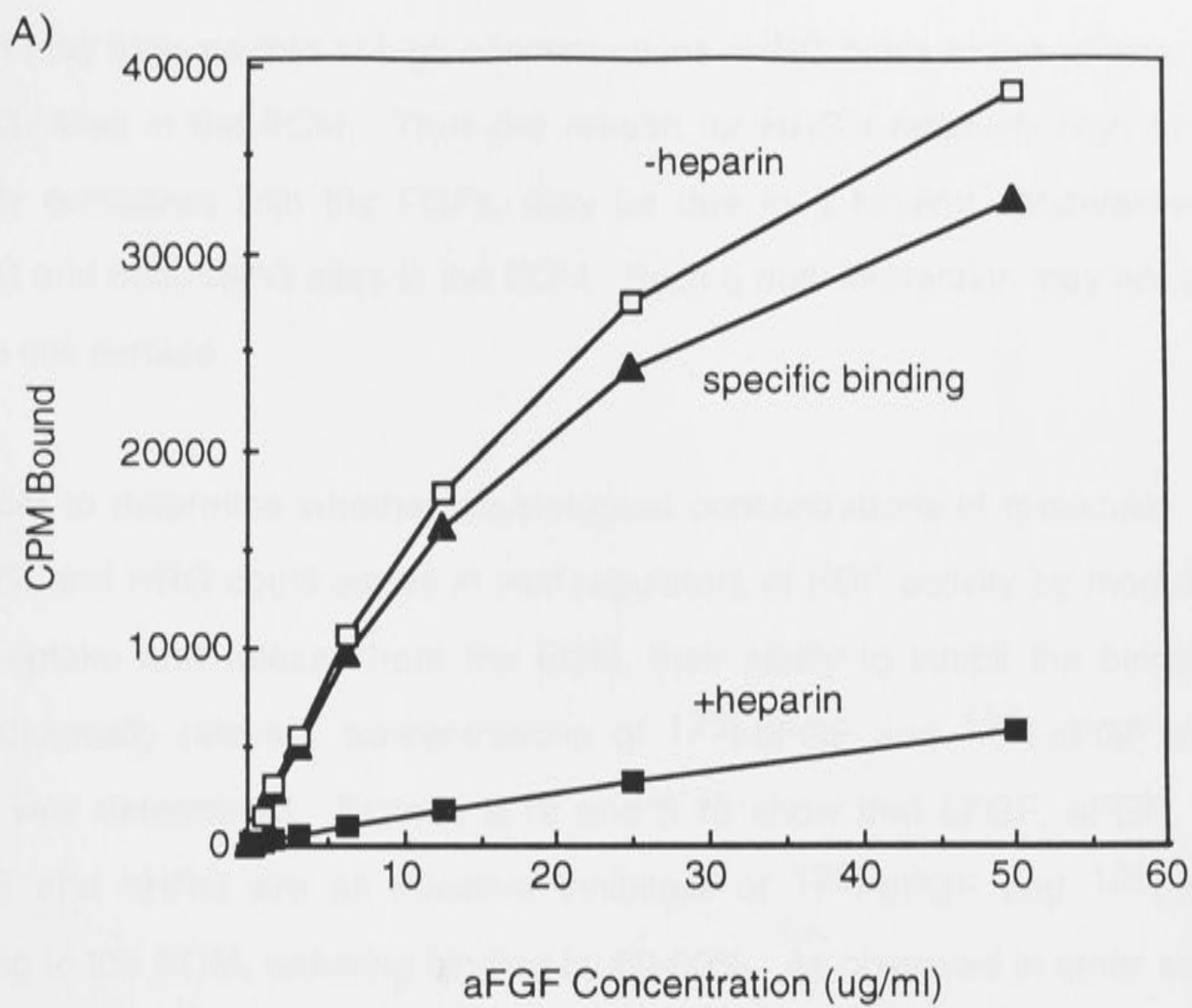




FIGURE 5.11: Ability of  $^{125}\text{I}$ -aFGF (A) and  $^{125}\text{I}$ -cHRG (B) to bind to bovine corneal endothelial cell extracellular matrix in the presence and absence of heparin (250ug/ml). Different concentrations of  $^{125}\text{I}$ -aFGF or  $^{125}\text{I}$ -cHRG were incubated in the presence and absence of heparin for 1 hour on ice, before being added to ECM-coated wells of a 96-well plate. After a further 2 hour incubation on ice, ECMs were washed free of unbound radioactivity and the ECMs detached by incubation with 4M guanidine-HCl/ 2% Triton X-100, overnight at 4°C. The amount of  $^{125}\text{I}$ -aFGF or  $^{125}\text{I}$ -cHRG bound to the ECM was determined and values represent the means of triplicates treatments. Standard errors of means were < 10 %.





which may indicate that at high concentrations cHRG binds to low affinity, non-HSPG, sites in the ECM. Thus the reason for HRG's relatively high binding affinity compared with the FGFs, may be due to it binding cooperatively to HSPG and non-HSPG sites in the ECM. Such a dual interaction may not occur at the cell surface.

In order to determine whether physiological concentrations of molecules such as PF4 and HRG could act as *in vivo* regulators of FGF activity by modulating FGF uptake and release from the ECM, their ability to inhibit the binding of physiologically relevant concentrations of  $^{125}\text{I}$ -bFGF and  $^{125}\text{I}$ -aFGF to the ECM was determined. Figures 5.12 and 5.13 show that bFGF, aFGF, PF4, cHRG and hHRG are all effective inhibitors of  $^{125}\text{I}$ -bFGF and  $^{125}\text{I}$ -aFGF binding to the ECM, reducing binding by 60-90%. As observed in other assays ATIII showed no inhibitory activity. The high degree of inhibition by heparin (70% with bFGF and 95% with aFGF) demonstrated the heparin/HS dependence of the binding assay. These results show that HRG and PF4, at concentrations approaching the physiological level, are able to substantially reduce the binding of  $^{125}\text{I}$ -aFGF (40ng/ml) and  $^{125}\text{I}$ -bFGF (2ng/ml) to the ECM.

#### **5.2.4. Inhibition of FGF Induced Mitogenesis by HRG**

Many attempts were made to determine whether cHRG could inhibit aFGF-induced 3T3 cell mitogenesis but unfortunately no inhibition of mitogenesis was observed. Subsequent binding inhibition studies with cHRG revealed that DMEM totally blocked cHRG binding to BALB/c 3T3 cells (Fig. 5.14 & Table 5.2). The inability of cHRG to bind to cells cultured in DMEM meant that mitogenic assays could not be performed in DMEM, the medium to which the 3T3 cells were accustomed. Furthermore, the cells were unable to proliferate and support aFGF-induced mitogenesis in the absence of fetal calf serum when cultured in F15, RPMI, and Medium199, media which were less inhibitory to

FIGURE 5.12: Ability of bFGF, aFGF, heparin , PF4, hHRG, cHRG and ATIII to inhibit the binding of  $^{125}\text{I}$ -bFGF (2ng/ml; 0.11nM) to bovine corneal endothelial cell ECM. ECMs were incubated with inhibitors for 1 hour on ice before the addition of  $^{125}\text{I}$ -bFGF which was followed by 2 hours incubation on ice. After washing away unbound  $^{125}\text{I}$ -bFGF, bound growth factor was released from the ECM by incubation with 4M guanidine HCl/ 2% Triton X-100 overnight at 4°C. The amount of  $^{125}\text{I}$ -bFGF released was determined and is presented as percent control binding, ie.,  $^{125}\text{I}$ -bFGF binding to ECM in the absence of inhibitor. Each value represents the mean of triplicate treatments  $\pm$  SEM.



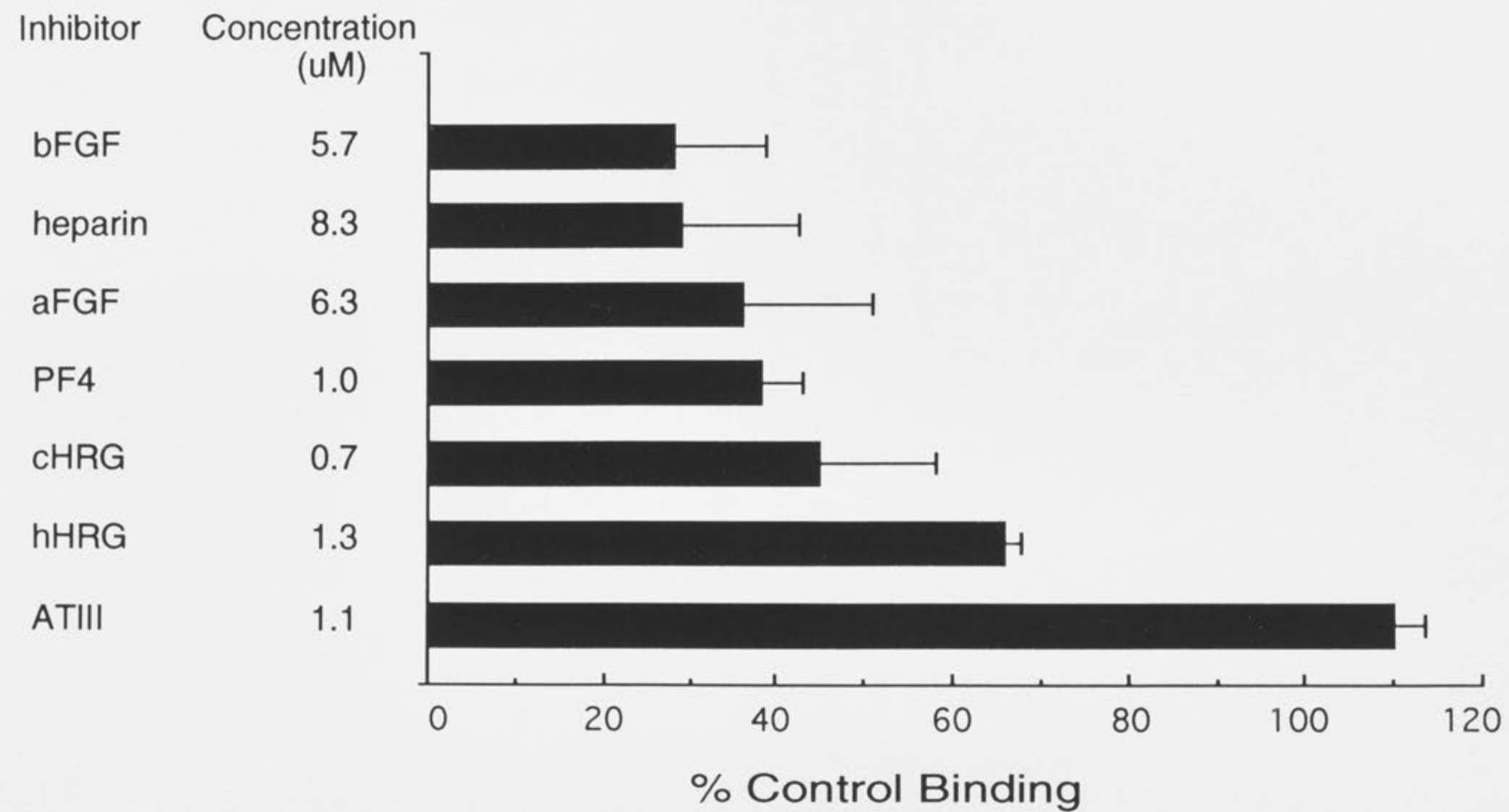
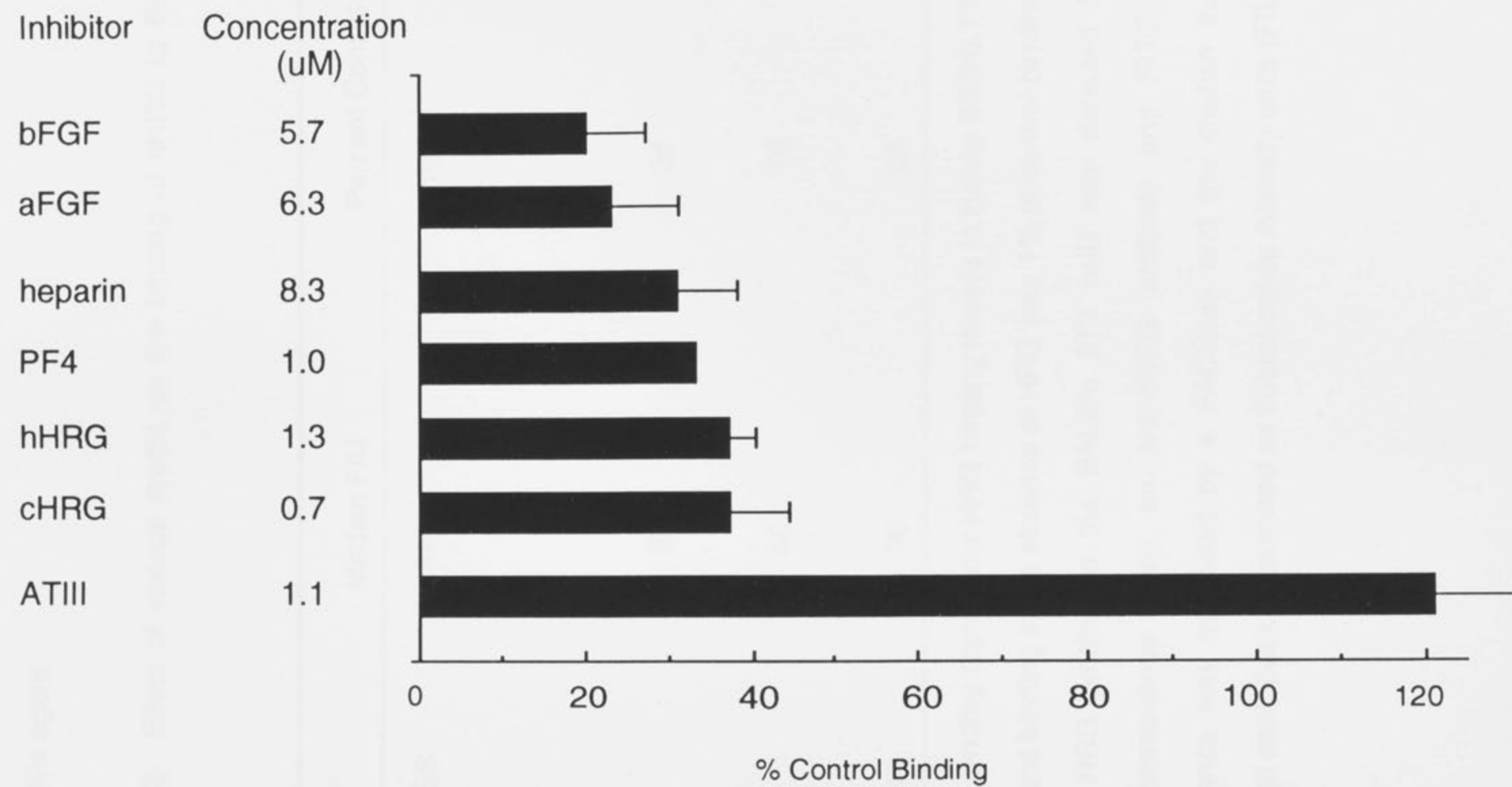


FIGURE 5.13: Ability of heparin, bFGF, PF4, hHRG, aFGF, cHRG and ATIII to inhibit the binding of  $^{125}\text{I}$ -aFGF (40ng/ml; 2.5nM) to bovine corneal endothelial cell ECM. Assay conditions and data presentation as described in the legend of Figure 5.12.





HRG binding (Table 5.2). Until the inhibitory component in DMEM is identified and removed we are unable to determine whether HRG can act as an anti-proliferative agent.

Table 5.2: Effect of various media on the binding of cHRG to BALB/c 3T3 cells<sup>a</sup>

Medium	Median FIU	Percent Control Binding
Hanks BSS	365	100
DMEM	7	2
199	93	25
F15	60	16
RPMI 1640	90	25

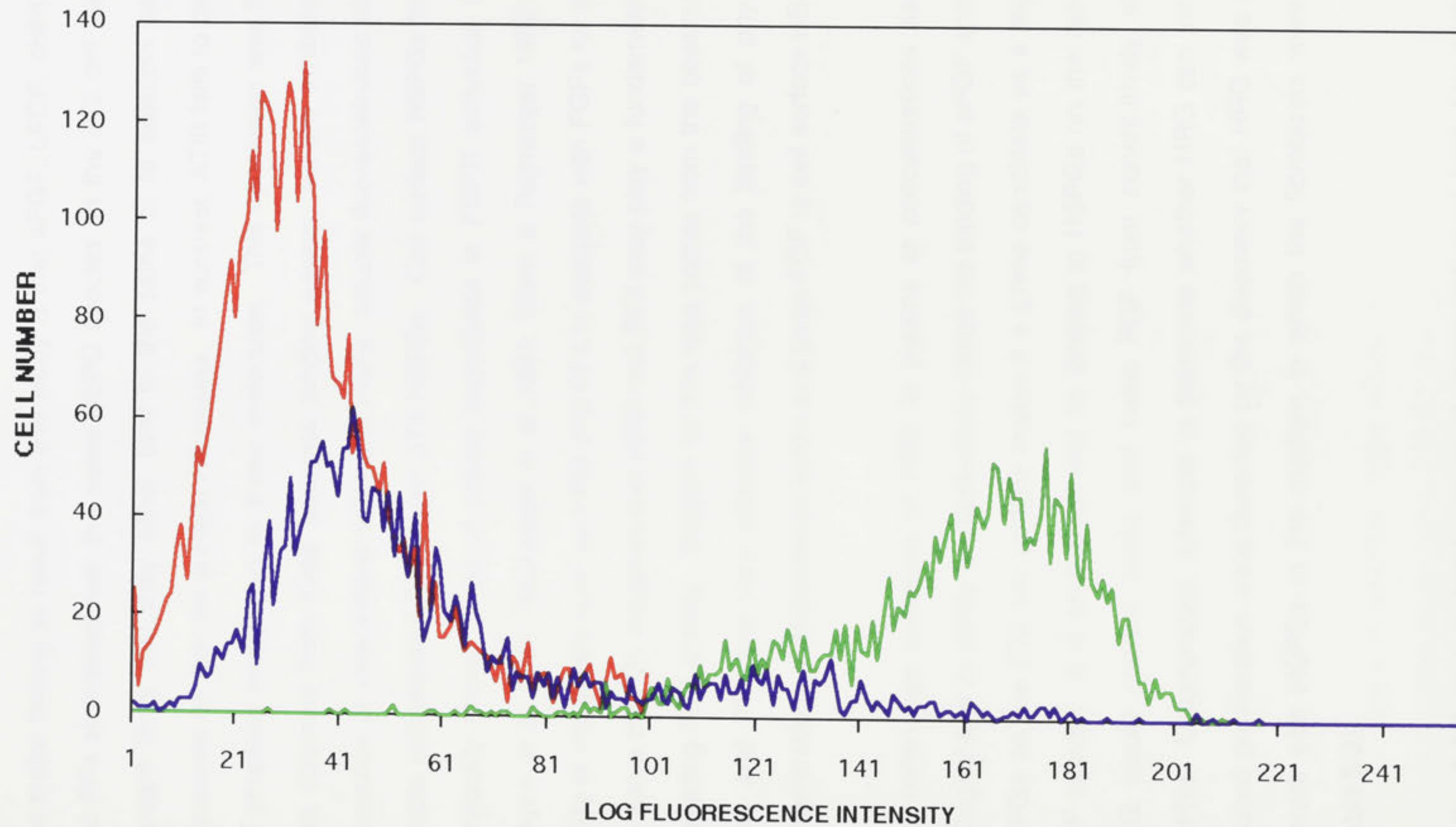
<sup>a</sup> Control binding represents HRG binding to cells in Hanks BSS/0.1%BSA.

Background binding in the absence of HRG was 1% of control binding.

Chicken HRG binding to the BALB/c 3T3 cells was detected by indirect immunofluorescence using an anti-cHRG antibody and FITC-Protein A. Fluorescence was quantified by a FACScan and the median fluorescence intensity of each peak determined as fluorescence intensity units (FIU).



FIGURE 5.14: FACScan profile showing the effect of DMEM on the binding of cHRG to 3T3 cells as measured by indirect immunofluorescence. The red line represents background binding, the blue line represents cHRG binding in the presence of DMEM and the green line represents control cHRG binding in Hanks BSS/0.1%BSA.





### 5.3. Discussion

The major finding to result from this study is that aFGF, bFGF, cHRG, hHRG and PF4 all interact with the same HSPG species on the 3T3 cell surface. It appears likely that they either bind to the same or to adjacent saccharide sequences on the cell surface HS chains. In contrast, ATIII fails to bind to the HS structure recognized by these molecules. This conclusion was based on data obtained from three separate binding assays, namely inhibition of the interaction of immobilized HRG and FGFs, soluble fluoresceinated cHRG and soluble radiolabeled FGFs, with 3T3 HSPGs. Cell surface HSPGs have been previously shown to be of critical importance in FGFR activation by FGFs (Yayon et al., 1991; Rapraeger et al., 1991; Olwin & Rapraeger 1992) and the ability of molecules such as HRG and PF4 to compete with FGFs for binding to the same HSPGs suggests that HRG and PF4 may play a fundamental role in regulating FGF activity. Support for this idea comes from the observation that HRG and PF4 are very effective inhibitors of the binding of physiological concentrations of radiolabeled acidic and basic FGF to cell surface HSPGs.

The continuous presence of HRG in plasma at concentrations as high as 100ug/ml and its ability to substantially inhibit the binding of aFGF and bFGF to HSPGs on the 3T3 cell surface makes it a prime candidate as a regulator of FGF activity. It is envisioned that by binding to HSPGs on the cell surface, HRG would prevent acidic and basic FGF from constitutively stimulating FGFRs. Unfortunately, attempts to determine whether HRG can inhibit FGF-induced proliferation were thwarted by the discovery that HRG was unable to interact with HSPGs in the medium in which the fibroblasts were cultured (Table 5.2).

PF4's distribution is highly localized, being released from blood platelets at sites of vascular injury, and is therefore less likely than HRG to act as a general



modulator of FGF activity. In addition PF4 has a very low plasma concentration (Lane et al.,1984), although, FGFs do play an important role in wound repair and, like PF4, are released at sites of vascular injury. At such sites PF4 may, on the one hand, aid the displacement of FGFs from the ECM, thus enabling the FGFs to bind to the cell surface and activate FGFRs and on the other hand, inhibit FGF-induced proliferation by masking cell surface HSPGs required for FGF action. PF4 has already been reported to have antiangiogenic properties, possibly as a result of its ability to inhibit endothelial cell proliferation (Taylor & Folkman 1982; Sharpe et al.,1990). The ability of PF4 to potently inhibit the binding of acidic and basic FGF to HSPGs on the fibroblast cell surface suggests that when added exogenously, PF4 inhibits FGF-induced proliferation by successfully competing with the FGFs for cell surface HSPGs. Although Maione et al.,(1990) found that PF4 can inhibit FGF-induced HUVE cell proliferation but not FGF-induced fibroblast proliferation, my results would tend to suggest that PF4 would be able to inhibit fibroblast proliferation by inhibiting FGF binding to the cell surface. Unfortunately I did not have sufficient supplies of PF4 to test its antiproliferative capacities. Nevertheless, these results do reinforce the idea that PF4's antiproliferative and antiangiogenic activities are due to its ability to inhibit FGFs from binding to the cell surface and activating their receptors.

Although the FGFs, HRGs and PF4 all interact with the same HSPG species, they differ in their affinities for them. Basic FGF and PF4 have the highest binding affinity, followed by cHRG and aFGF which appear to exhibit approximately equal affinity, and hHRG has the lowest affinity. The inhibition data presented in this chapter, using three different binding assays, confirmed that aFGF has a substantially lower affinity (approx. 5 fold) for 3T3 cell surface HSPG than bFGF (see section 3.2.1). The data is also in agreement with D.A. Lane's observation (unpublished) that HRG is eluted from immobilized heparin with lower concentrations of NaCl than PF4 suggesting that PF4 has a higher



affinity for heparin than HRG. This may be due to PF4's tetrameric structure and its ability to form a complex with up to four heparin-like molecules simultaneously (Marshall et al., 1984) while human HRG probably has only one heparin binding site per molecule (Burch et al., 1987). Cross blocking studies also indicated that ATIII did not interact with the HS sequences on 3T3 cells recognized by the FGFs and HRGs. Lane et al., (1986) also found that both PF4 and HRG interact with HS sequences in addition to the pentasaccharide sequence with which ATIII binds.

Acidic and basic FGF are known to be localized to basement membranes (BM), both growth factors having been identified in ECMs deposited by cultured myoblasts (Weiner & Swain 1989) and endothelial cells (Baird & Ling 1987; Vlodavsky et al., 1987a). Basic FGF is also present in BMs of rat fetus (Gonzalez et al., 1990), bovine cornea (Folkman et al., 1988) and human blood vessels (Cordon-Cardo et al., 1990). Scatchard analysis of heparin-inhibitable binding of acidic and basic FGF to the ECM suggests that both FGFs bind to HSPG in the ECM with considerably lower affinity ( $K_d$ s of  $2.7 \pm 0.5 \mu\text{M}$  and  $1.7 \pm 0.2 \mu\text{M}$ , respectively) than they are thought to bind to HSPGs on the cell surface ( $K_d$  2-10nM) (Moscatelli 1987). Thus, FGFs may be able to pass from low affinity receptors in the ECM to relatively higher affinity HSPGs on the cell surface according to kinetic equilibrium. The ability of HRG to also bind to the ECM in a heparin-inhibitable manner suggests that HRG may also be able to modulate FGF activity by regulating its release from the matrix.

Although matrix bound FGF is known to be biologically active, the presence of FGF in BMs *in vivo* does not appear to stimulate the overlying endothelial and epithelial cells to proliferate (D'Amore 1990a). It has therefore been suggested that while bound to HSs in the ECM/BM, FGFs are not accessible to their cell surface receptors. Indeed, the ECM/BM has been proposed to act as a physiological reservoir for growth factors holding them in a stable and protected



form until they are required to bind to and activate FGFRs on the cell surface. This would also explain how FGFs are prevented from acting on and stimulating vascular endothelium continuously. It is not clear though, how FGFs are released from the ECM when they are required to stimulate cell proliferation. Current hypotheses suggest that HS-bound matrix-associated FGFs might be released by locally high concentrations of specific HS-degrading enzymes (heparanases) (Flaumenhaft et al., 1989) or by locally high concentrations of heparin which may originate from degranulating mast cells (Jansson et al., 1975; Yurt et al., 1977). In support of the former possibility, Ishai-Michaeli et al., (1990) have demonstrated the release of active bFGF from the ECM by intact platelets, neutrophils and lymphoma cells, all of which express heparanase activity. It has also been proposed that physical damage to the matrix occurring during injury, or damage occurring during tissue remodeling, could result in the release of FGFs from their storage sites (Folkman et al., 1988). The data presented in this chapter suggest that an alternative means by which FGFs could be released from the ECM would be if HRG or PF4 were to displace them from their HSPG binding sites in the ECM, making FGF available for binding to HSPGs on the cell surface.

In conclusion, studies described in this chapter have demonstrated that PF4 and HRG are potentially important regulators of FGF action by competing for the same or closely associated HS motifs on HSPGs. These molecules could regulate FGF action in both a positive and negative sense, on the one hand by displacing FGF from ECM/BM and making these FGFs available to responsive cells, and on the other hand by masking HSPGs on responsive cells and preventing FGFR activation.



#### 5.4. Summary

Recent studies have shown that FGFs need to interact with cell surface HSPG in order to bind to and activate FGFR. In this chapter, three major heparin-binding proteins, histidine-rich glycoprotein (HRG), antithrombin III (ATIII) and platelet factor 4 (PF4) were tested for their ability to act as modulators of FGF activity by competing with the FGFs for cell surface HSPGs. HRG and ATIII are present at high concentrations in plasma whereas PF4 is released locally at high concentrations by degranulating platelets. HRG from both chicken and human, and human PF4 were demonstrated to compete with each other and with acidic and basic FGF for binding to BALB/c 3T3 cell surface HSPGs, whereas ATIII did not compete. Thus HRG, PF4, aFGF and bFGF all interact with the same HS chains on the 3T3 cell surface, either binding to the same or to adjacent saccharide sequences on the chains. In terms of their relative binding affinity for cell surface HSPGs, the hierarchy was shown to be  $PF4 \geq bFGF > aFGF = cHRG > hHRG$ . HRG was also shown to bind to ECMs, originating from bovine corneal endothelial cells, in a heparin-inhibitable manner. Indeed both HRG and PF4 were shown to effectively inhibit the binding of  $^{125}I$ -aFGF and  $^{125}I$ -bFGF to ECMs, at concentrations thought to occur *in vivo*. Based on these findings, it is proposed that HRG and PF4 may act as positive regulators of FGF activity by displacing FGF from the ECM or BM and making FGF available to responsive cells. Alternatively, they could act as negative regulators by masking HSPGs on responsive cells and preventing FGFR activation.



## Chapter 6

### Final Discussion

In this thesis several aspects of the roles of soluble heparin and cell surface HSPGs in the functioning of acidic and basic FGF were examined. The experimental results obtained may be divided into the three sections, considered below.

#### 6.1. Role of Cell Surface HSPGs in Binding of FGFs to the FGFR

It is now clear that the binding of FGF to heparin or HSPG is an essential requirement for activation of the FGF receptor by FGFs (Yayon et al 1991; Rapraeger et al., 1991; Olwin & Rapraeger 1992) and the experimental work presented in this thesis confirms and expands upon this finding. Two models have been proposed to explain the role of HSPG/heparin in the interaction of bFGF with the FGFR. Yayon et al., (1991) have proposed that the interaction of HSPG or heparin with bFGF produces a conformational change in bFGF which allows it to interact with the FGFR (Fig. 1.8). In contrast, Nugent & Edelman (1992) have proposed that the formation of a ternary complex either between bFGF, FGFR and HSPG or between bFGF, FGFR, heparin and heparin binding sites on the cell surface, is required to produce an interaction of sufficiently high avidity for receptor activation to occur (Fig. 1.9). In the light of a recent finding by Kan et al., (1993) that FGFR1 possesses a heparin binding site which is essential for FGF binding to FGFR1, a ternary complex could also result from an FGF/heparin complex crosslinking heparin- and FGF-binding sites on the same FGFR.

In this thesis, evidence has been presented which suggests that both a heparin/HSPG-induced conformational change in the growth factor and the formation of a ternary complex may be required for the interaction of FGF with the FGFR. Furthermore, I have obtained evidence to suggest that aFGF and



bFGF have different requirements for binding to FGFR1 which may have implications for understanding how cells and tissues can selectively respond to individual members of the FGF family. I will now summarize the evidence I have obtained which supports the ternary complex model of FGFR activation. Firstly, in chapter 3, bFGF was shown to dissociate from the FGFR at least 20-fold slower in the presence of HSPGs than in their absence which confirms the finding of Nugent & Edelman (1992) and suggests that HSPGs are an essential requirement for "high avidity" binding of bFGF to FGFR. It cannot be assumed that the rate of FGF dissociation from the FGFR would change as a result of an increase in affinity of FGF for its receptor, arising from a conformational change in the growth factor. In fact, there is considerable evidence showing that the affinity constant of a receptor for a ligand applies only at equilibrium and is independent of the dissociation and association rate of the receptor-ligand interaction (Williams 1991). Thus, the formation of a ternary complex as a result of crosslinking of the FGFR and HSPG by FGF is more likely to stabilize the binding of the growth factor to its receptor and prolong the period that it is bound to the FGFR, ie., decrease the dissociation rate. Secondly, it was shown that aFGF, in the presence of cell surface HSPGs, dissociates from the FGFR at least 10-fold faster than does bFGF. This result, together with the demonstration that aFGF has an approximately 5-fold lower affinity for cell surface HSPGs than bFGF, suggests that at low concentrations, aFGF is not able to utilize 3T3 cell surface HSPGs to form ternary complexes as effectively as does bFGF.

Further support for the ternary complex model comes from comparative studies performed with heparin and CR-heparin in chapter 4 which showed that CR-heparin was much less effective than native heparin at enabling aFGF to form a stable interaction with the FGFR. The ability of CR-heparin to bind aFGF equally as well as does heparin and to protect aFGF from digestion by trypsin, makes it unlikely that CR-heparin is unable to induce a conformational change



in the molecule. It seems more plausible that CR-heparin fails to enable the formation of a stable ternary complex. In addition, the demonstration that CR-heparin is only able to bind to 1/3 the number of heparin receptors on the cell surface to which heparin binds, suggests that CR-heparin may be less able to form ternary complexes with FGF, FGFR and heparin binding sites, as can occur with heparin. There may in fact be specific heparin binding receptors on the cell surface to which CR-heparin does not bind which are critical for the formation of such ternary complexes with the FGFRs.

Furthermore, preliminary data from the laboratory of David Ornitz suggests that CR-heparin has a considerably lower affinity than heparin for the heparin binding site on the FGFR. In the absence of FGF, CR-heparin was at least 10-fold less able than heparin to bind to the soluble form of murine FGFR1 and approximately 25-fold less able to bind to the soluble form of murine FGFR3. One would predict that CR-heparin's poor ability to interact with the heparin binding site on FGFR1 would prevent the formation of stable ternary complexes between FGF and the FGFR. Indeed, in chapter 4, CR-heparin was observed to have a reduced ability to enhance the binding of aFGF to the FGFR on heparinase treated cells. Moreover, in the soluble receptor binding assay, CR-heparin was totally unable to enhance the binding of bFGF to FGFR1 and only partially able to restore the binding of aFGF to FGFR1.

Finally, although CR-heparin was completely unable to enhance the binding of bFGF to FGFR1 in the soluble receptor assay, preliminary results from David Ornitz's laboratory using BAF3 cells transfected with mFR1, show that CR-heparin does result in some enhancement of bFGF-induced mitogenesis but not as much as with native heparin which indicates that other cell surface molecules (ie., heparin binding molecules) may be involved in the formation of ternary complexes.



The strongest evidence I obtained in favour of heparin inducing a conformational change in FGF comes from my observation that heparin increased the net amount of acidic and basic FGF bound to the FGFR on heparinase treated cells without affecting FGF association or dissociation rates (see chapter 3). If heparin was replacing the role of HSPGs in the formation of a ternary complex, one would have expected heparin to produce a decrease in the dissociation rate of FGF from the FGFR. However, the increase in the net amount of FGF bound to the FGFR in the presence of heparin, suggests that heparin may have induced a conformational change in the growth factor which increased its affinity for the FGFR, thereby enabling more receptors to be engaged.

Further evidence in favour of a conformational change occurring in aFGF, but not bFGF, comes from the results of the soluble receptor assay (see chapter 4) in which CR-heparin partially restored the binding of aFGF to FGFR1 but totally failed to enhance the binding of bFGF to the same receptor. Since CR-heparin was found to bind to acidic and basic FGF equally as well as native heparin, this inactivity is not due to an inability to bind the growth factors. It may indicate though, that CR-heparin was able to induce a conformational change in aFGF, but not facilitate the formation of a ternary complex, thus allowing some binding of aFGF to its receptor. In contrast, CR-heparin was unable to induce either a conformational change or the formation of a ternary complex with bFGF.

In conclusion, it appears that HSPGs and heparin are able to mediate both a conformational change in the growth factor and the formation of a ternary complex and that both are important in the interaction of FGF with FGFR1. It is highly likely that the relative balance of conformational change versus ternary complex formation may vary with each FGFR and between FGFs and a given FGFR. In the case of FGFR1, I propose that for optimal binding of aFGF to



FGFR1 to occur, a heparin-induced conformational change in aFGF must be followed by the formation of a ternary complex, whereas for the binding of bFGF to FGFR1, only the formation of a ternary complex is required.

## **6.2. Mode of Potentiation of aFGF Induced Mitogenic Activity by Heparin**

For the last ten years it has been known that heparin potentiates the biological activity of aFGF *in vitro* (Thornton et al., 1983; Orlidge & D'Amore 1986; Schreiber et al., 1985), although the mechanism by which it does so is not fully understood. In chapter 3 of this thesis, I have confirmed that heparin potentiates aFGF-induced mitogenesis on BALB/c 3T3 cells and HUVE cells to a significantly greater extent than it does bFGF-induced mitogenesis. Moreover, in the absence of exogenously added heparin, aFGF's potency as a mitogen *in vitro* is markedly less than that of bFGF. It is not until heparin is added into the culture with aFGF that aFGF's potency increases to levels comparable with that of bFGF.

From my data, it appears that the difference in potency of aFGF and bFGF is due to the relative lower binding affinity of aFGF for the 3T3 cell surface HSPGs, ie., approximately a 5 fold difference based on my measurements. By having a higher affinity for cell surface HSPGs, bFGF is able to activate FGFRs at much lower concentrations than aFGF. On the other hand, aFGF very likely has a higher affinity for heparin than it does for 3T3 HSPGs and is thus able to utilize heparin more effectively in interacting with the FGFR (whether a conformational change or ternary complex is required). Thus heparin increases the number of productive complexes formed between aFGF and its FGFR at limiting concentrations of aFGF, resulting in a potentiation of aFGF's mitogenic activity. Therefore, providing that the correct FGFR is expressed on the cell surface, FGF induced mitogenic activity appears to be directly related to the affinity of the growth factor for cell surface HSPGs. Indeed, FGF activity during murine neural precursor cell development *in vivo* has been shown to be



regulated in part, by the affinity of aFGF and bFGF for a particular species of endogenous HSPG (Nurcombe et al., 1993). HSPGs from E9 which bind bFGF at least four times better than aFGF were approximately seven times better at stimulating neural epithelial cell division with bFGF than with aFGF whereas the E11 HSPGs which bind aFGF better, were four times better at stimulating cell division with aFGF than with bFGF.

### **6.3. Inhibition of Binding of FGFs to Cell Surface HSPGs by Heparin Binding Proteins**

Acidic and basic FGF are known to have a role in various pathological processes. For example, FGFs have been isolated from cultured tumour cells and from tumour tissue (reviewed by Gospodarowicz 1987b) and are thought to stimulate tumour cell growth as autocrine growth factors and as tumour angiogenic factors (Gospodarowicz et al., 1987b; Schweigerer et al., 1987b; Folkman & Klagsbrun 1987; Burgess & Maciag 1989; Nagao & Nishikawa 1989). In addition, FGFs may promote solid tumour growth by vascularizing the invading tissue (Folkman & Klagsbrun 1987), promote arthritis by stimulating the invasion of new capillary blood vessels into joints, and in diabetes, stimulate capillaries in the retina to invade the vitreous, resulting in blindness (Folkman 1987). Compounds which can selectively inhibit the activation of FGFRs by FGFs could be important in the prevention of such angiogenesis-related pathologies. Most attempts at developing inhibitors have focussed on inhibiting the interaction of FGF with the FGFR. An alternative means of inhibiting activation of the FGFR would be by inhibiting the interaction between FGF and cell surface HSPGs.

In chapter 5, I have examined several naturally occurring heparin binding proteins, namely HRG, PF4 and ATIII, for their ability to disrupt the interaction between FGFs and HSPGs. Interestingly, it was found that HRG and PF4, but not ATIII, interact with the same HSPG species on the 3T3 cell surface as do



acidic and basic FGF. Acidic and basic FGF also interact with the same HSPGs as each other. However, it is not clear whether aFGF, bFGF, cHRG, hHRG and PF4 all bind to the same, or to adjacent saccharide sequences on the HS chains. Furthermore, HRG and PF4 were found to be very effective inhibitors of the binding of radiolabeled acidic and basic FGF, at physiological concentrations, to HSPGs situated both on the 3T3 cell surface and in bovine corneal endothelial cell ECMs. Unfortunately technical problems prevented me from testing HRG and PF4 for their ability to inhibit FGF-induced proliferation. However, the observations described above, suggest that HRG and PF4 may actually play a role in regulating FGF activity *in vivo*. It is proposed that HRG, which is present in plasma continuously and at concentrations as high as 100ug/ml, by binding to HSPGs on the cell surface would prevent acidic and basic FGF from constitutively stimulating FGFRs. Very little is currently known about how FGFs are kept in abeyance until they are required to activate their receptors. On the other hand, PF4 tends to be localized at sites of vascular injury. However, at such sites it may be able to aid the displacement of FGFs from the ECM, thus enabling them to bind to cell surface HSPGs and activate the FGFR. A similar situation may apply for HRG. Alternatively, PF4 may act as an inhibitor of FGF-induced proliferation by masking cell surface HSPGs required for FGF action.

#### **6.4. Future Work**

A number of interesting experiments need to be performed to clarify some of the observations reported upon in this thesis. Of particular importance is the identity and action of the component in DMEM which is inhibitory to HRG action. Once identified, this component could be removed from the culture medium and HRG tested for its ability to act as an antiproliferative agent. A related study would be to test the ability of HRG to bind to the two forms of differentially glycosylated HSPG, isolated by Nurcombe et al.,(1993) which exhibit specific affinities for aFGF or bFGF. If HRG does bind to these, it would



be of interest to test the ability of cHRG to displace FGFs from these HSPGs which should give some indication as to whether HRG could actually displace FGFs from cell surface HSPGs *in vivo*. In addition, experiments could be attempted to determine whether HRG has the ability to inhibit the binding of heparin/HSPG to the heparin binding site on FGFR1 and FGFR3 in the soluble receptor binding assay. If so, it would suggest that HRG has the potential to act as an inhibitor of FGFR activation. Eventually, it would be interesting to determine the particular saccharide sequence on heparin/HSPG with which HRG interacts and compare it to that with which the FGFs bind (Turnbull et al 1992; Habuchi et al 1992).

Further work with CR-heparin and its fragments, in collaboration with Dr Ornitz, may help to clarify the different requirements that acidic and basic FGF appear to have for binding to FGFR1, in the soluble receptor binding assay. It would also be interesting to compare the requirements of the two growth factors for binding to some of the other FGFRs with a view to understanding the role of conformational change versus ternary complex formation in determining the specificity of the receptor/ligand interaction obtained.

A longer term study would be the examination of the role of FGFs and other growth factors, and the expression of their receptors, during angiogenesis. Many of the pathologies in which FGFs have been implicated appear to be related to stimulation of blood vessel growth and therefore, an area of considerable potential is the development of inhibitors of angiogenesis and application of them to inhibition of tumour growth. One strategy would be to develop and screen for, sugar fragments able to inhibit angiogenesis. Sugar fragments unable to bind FGFs but able to bind to and block the heparin binding site on the FGFR would be expected to inhibit FGF activation of the FGFR as would sugar fragments able to bind FGFs, but unable to interact with the heparin binding site on the FGFR. Whatever the outcome of the studies

outlined briefly above, it is clear from my work and the work of other laboratories that HSPGs play a key role in the functioning of many heparin-binding growth factors.



## References

- Abraham, J.A., Mergia, A., Whang, J.L., Tumolo, A., Friedman, J., Hjerrild, K.A., Gospodarowicz, D., and Fiddes, J.C. (1986a): Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. *Science*, 233:545-548.
- Abraham, J.A., Whang, J.L., Tumolo, A., Mergia, A., and Fiddes, J.C. (1986b): Human basic fibroblast growth factor: nucleotide sequence, genomic organization, and expression in mammalian cells. *Cold Spring Harb. Symp. Quant. Biol.*, 51 Pt 1:657-668.
- Abraham, J.A., Whang, J.L., Tumolo, A., Mergia, A., Friedman, J., Gospodarowicz, D., and Fiddes, J.C. (1986c): Human basic fibroblast growth factor: nucleotide sequence and genomic organization. *EMBO J.*, 5:2523-2528.
- Adnane, J., Gaudray, P., Simon, M.P., Simony-Lafontaine, J., Jeanteur, P., and Theillet, C. (1989): Proto-oncogene amplification and human breast tumor phenotype. *Oncogene*, 4:1389-1395.
- Akiyama, S.K. and Yamada, K.M. (1985): Synthetic peptides competitively inhibit both direct binding to fibroblasts and functional biological assays for the purified cell-binding domain of fibronectin. *J. Biol. Chem.*, 260:10402-10405.
- Albrecht-Buehler, G. (1977): The phagocytic tracks of 3T3 cells. *Cell*, 11:395-404.
- Amalric, F., Baldin, V., Bosc-Bierne, I., Bugler, B., Couderc, B., Guyader, M., Patry, V., Prats, H., Roman, A.M., and Bouche, G. (1991): Nuclear translocation of basic fibroblast growth factor. *Ann. N.Y. Acad. Sci.*, 638:127-138.
- Amaya, E., Musci, T.J., and Kirschner, M.W. (1991): Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell*, 66:257-270.
- Armelin, H.A. (1973): Pituitary extracts and steroid hormones in the control of 3T3 cell growth. *Proc. Natl. Acad. Sci. U.S.A.*, 70:2702-2706.
- Arruti, C. and Courtois, Y. (1978): Morphological changes and growth stimulation of bovine epithelial lens cells by a retinal extract in vitro. *Exp. Cell Res.*, 117:283-292.
- Baird, A. and Bohlen, P. (1990): *Peptide Growth Factors*. Springer-Verlag, New York, NY.
- Baird, A., Esch, F., Bohlen, P., Ling, N., and Gospodarowicz, D. (1985a): Isolation and partial characterization of an endothelial cell growth factor from the bovine kidney: homology with basic fibroblast growth factor. *Regul. Pept.*, 12:201-213.



Baird, A., Esch, F., Gospodarowicz, D., and Guillemin, R. (1985b): Retina- and eye-derived endothelial cell growth factors: partial molecular characterization and identity with acidic and basic fibroblast growth factors. *Biochemistry*, 24:7855-7860.

Baird, A., Mormede, P., and Bohlen, P. (1985c): Immunoreactive fibroblast growth factor in cells of peritoneal exudate suggests its identity with macrophage-derived growth factor. *Biochem.Biophys.Res.Comm.*, 126:358-364.

Baird, A., Esch, F., Mormede, P., Ueno, N., Ling, N., Bohlen, P., Ying, S.Y., Wehrenberg, W.B., and Guillemin, R. (1986): Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues. *Recent.Prog.Horm.Res.*, 42:143-205.

Baird, A. and Ling, N. (1987): Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implications for a role of heparinase-like enzymes in the neovascular response. *Biochem.Biophys.Res.Comm.*, 142:428-435.

Baird, A., Schubert, D., Ling, N., and Guillemin, R. (1988): Receptor- and heparin-binding domains of basic fibroblast growth factor. *Proc.Natl.Acad.Sci.U.S.A*, 85:2324-2328.

Baird, A. and Walicke, P.A. (1989): Fibroblast growth factors. *Br.Med.Bull.*, 45:438-452.

Baldin, V., Roman, A.M., Bosc-Bierne, I., Amalric, F., and Bouche, G. (1990): Translocation of bFGF to the nucleus is G1 phase cell cycle specific in bovine aortic endothelial cells. *EMBO J.*, 9:1511-1517.

Bame, K.J., Lidholt, K., Lindahl, U., and Esko, J.D. (1991): Biosynthesis of heparan sulfate. Coordination of polymer-modification reactions in a Chinese hamster ovary cell mutant defective in N-sulfotransferase. *J.Biol.Chem.*, 266:10287-10293.

Bar-Ner, M., Mayer, M., Schirmacher, V., and Vlodavsky, I. (1986): Involvement of both heparanase and plasminogen activator in lymphoma cell-mediated degradation of heparan sulfate in the subendothelial extracellular matrix. *J.Cell Physiol.*, 128:299-306.

Barber, A.J., Kaser-Glanzmann, R., Jakabova, M., and Luscher, E.F. (1972): Characterization of a chondroitin 4 -sulfate proteoglycan carrier for heparin neutralizing activity (platelet factor 4 ) released from human blood platelets. *Biochim.Biophys.Acta*, 286:312-329.

Barritault, D., Arruti, C., and Courtois, Y. (1981): Is there a ubiquitous growth factor in the eye? Proliferation induced in different cell types by eye-derived growth factors. *Differentiation.*, 18:29-42.



Barzu, T., Van-Rijn, J.L., Petitou, M., Molho, P., Tobelem, G., and Caen, J.P. (1986a): Endothelial binding sites for heparin. Specificity and role in heparin neutralization. *Biochem.J.*, 238:847-854.

Barzu, T., vanRijn, J.L.M.L., Rosenbaum, J., and Tobelem, G. (1986b): Endothelial cell reactions with heparin. *The Medical Journal of Australia*, 144:15-17.

Barzu, T., Lormeau, J.-C., Petitou, M., Michelson, S., and Choay, J. (1989): Heparin-derived oligosaccharides: affinity for acidic fibroblast growth factor and effect on its growth-promoting activity for human endothelial cells. *J.Cell.Physiol.*, 140:538-548.

Bashkin, P., Doctrow, S., Klagsbrun, M., Svahn, C.M., Folkman, J., and Vlodavsky, I. (1989): Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparitinase and heparin-like molecules. *Biochemistry*, 28:1737-1743.

Belford, D.A., Hendry, I.A., and Parish, C.R. (1992): Ability of different chemically modified heparins to potentiate the biological activity of heparin-binding growth factor 1: lack of correlation with growth factor binding. *Biochemistry*, 31:6498-6503.

Belford, D.A., Hendry, I.A., and Parish, C.R. (1993): Investigation of the ability of several naturally occurring and synthetic polyanions to bind to, and potentiate the biological activity of acidic fibroblast growth factor. *J.Cell.Physiol.*, (in press)

Bellot, F., Crumley, G., Kaplow, J.M., Schlessinger, J., Jaye, M., and Dionne, C.A. (1991): Ligand-induced transphosphorylation between different FGF receptors. *EMBO J.*, 10:2849-2854.

Bennett, M.V., Barrio, L.C., Bargiello, T.A., Spray, D.C., Hertzberg, E., and Saez, J.C. (1991): Gap junctions: new tools, new answers, new questions. *Neuron*, 6:305-320.

Bernard, O., Li, M., and Reid, H.H. (1991): Expression of two different forms of fibroblast growth factor receptor 1 in different mouse tissues and cell lines. *Proc.Natl.Acad.Sci.U.S.A.*, 88:7625-7629.

Bernfield, M. and Sanderson, R.D. (1990): Syndecan, a developmentally regulated cell surface proteoglycan that binds extracellular matrix and growth factors. *Philos.Trans.R.Soc.Lond.Biol.*, 327:171-186.

Bitter, I. and Muir, H.M. (1962): A modified uronic acid carbazole reaction. *Anal.Biochem.*, 4:330-334.

Bjork, I. and Nordenman, B. (1976): Acceleration of the reaction between thrombin and antithrombin III by non-stoichiometric amounts of heparin. *Eur.J.Biochem.*, 68:507-511.



Bjornsson, T.D., Dryjski, M., Tluczek, J., Mennie, R., Ronan, J., Mellin, T.N., and Thomas, K.A. (1991): Acidic fibroblast growth factor promotes vascular repair. *Proc.Natl.Acad.Sci.U.S.A*, 88:8651-8655.

Black, E.G., Logan, A., Davis, J.R., and Sheppard, M.C. (1990): Basic fibroblast growth factor affects DNA synthesis and cell function and activates multiple signalling pathways in rat thyroid FRTL-5 and pituitary GH3 cells. *J.Endocrinol.*, 127:39-46.

Bohlen, P., Baird, A., Esch, F., Ling, N., and Gospodarowicz, D. (1984): Isolation and partial molecular characterization of pituitary fibroblast growth factor. *Proc.Natl.Acad.Sci.U.S.A*, 81:5364-5368.

Bohlen, P., Esch, F., Baird, A., Jones, K.L., and Gospodarowicz, D. (1985): Human brain fibroblast growth factor. Isolation and partial chemical characterization. *FEBS Lett.*, 185:177-181.

Bouche, G., Gas, N., Prats, H., Baldin, V., Tauber, J.-P., Teissie, J., and Amalric, F. (1987): Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing Go-G1 transition. *Proc.Natl.Acad.Sci.USA*, 84:6770-6774.

Bovi, P.D. and Basilico, C. (1987): Isolation of a rearranged human transforming gene following transfection of Kaposi sarcoma DNA. *Proc.Natl.Acad.Sci.U.S.A*, 84:5660-5664.

Brigstock, D.R., Sasse, J., and Klagsbrun, M. (1991): Subcellular distribution of basic fibroblast growth factor in human hepatoma cells. *Growth Factors*, 4:189-196.

Brown, K.D., Blakeley, D.M., and Brigstock, D.R. (1989): Stimulation of polyphosphoinositide hydrolysis in Swiss 3T3 cells by recombinant fibroblast growth factors. *FEBS Lett.*, 247:227-231.

Brunner, G., Gabrilove, J., Rifkin, D.B., and Wilson, E.L. (1991): Phospholipase C release of basic fibroblast growth factor from human bone marrow cultures as a biologically active complex with a phosphatidylinositol-anchored heparan sulfate proteoglycan. *J.Cell Biol.*, 114:1275-1283.

Bugler, B., Amalric, F., and Prats, H. (1991): Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. *Mol.Cell Biol.*, 11:573-577.

Burch, M.K., Blackburn, M.N., and Morgan, W.T., (1987): Further characterization of the interaction of histidine-rich glycoprotein with heparin: evidence for the binding of two molecules of histidine-rich glycoprotein by high molecular weight heparin and for the involvement of histidine residues in heparin binding. *Biochem.*, 26:7477-7482.

Burgess, W.H. and Maciag, T. (1989): The heparin-binding (fibroblast) growth factor family of proteins. *Annu.Rev.Biochem.*, 58:575-606.



Burgess, W.H., Dionne, C.A., Kaplow, J., Mudd, R., Friesel, R., Zilberstein, A., Schlessinger, J., and Jaye, M. (1990a): Characterization and cDNA cloning of phospholipase C-gamma, a major substrate for heparin-binding growth factor 1 (acidic fibroblast growth factor)-activated tyrosine kinase. *Mol. Cell Biol.*, 10:4770-4777.

Burgess, W.H., Shaheen, A.M., Ravera, M., Jaye, M., Donohue, P.J., and Winkles, J.A. (1990b): Possible dissociation of the heparin-binding and mitogenic activities of heparin-binding (acidic fibroblast) growth factor-1 from its receptor-binding activities by site-directed mutagenesis of a single lysine residue. *J. Biol. Chem.*, 111:2129-2138.

Burgess, W.H., Shaheen, A.M., Hampton, B., Donohue, P.J., and Winkles, J.A. (1991): Structure-function studies of heparin-binding (acidic fibroblast) growth factor-1 using site-directed mutagenesis. *J. Cell. Biochem.*, 45:131-138.

Burwen, S.J. and Jones, A.L. (1987): The association of polypeptide hormones and growth factors with the nuclei of target cells. *TIBS*, 12:159-162.

Canalis, E., Lorenzo, J., Burgess, W.H., and Maciag, T. (1987): Effects of endothelial cell growth factor on bone remodelling in vitro. *J. Clin. Invest.*, 79:52-58.

Canalis, E. and Lian, J.B. (1988): Effects of bone associated growth factors on DNA, collagen and osteocalcin synthesis in cultured fetal rat calvariae. *Bone*, 9:243-246.

Carey, D.J. and Evans, D.M. (1989): Membrane anchoring of heparan sulfate proteoglycans by phosphatidylinositol and kinetics of synthesis of peripheral and detergent-solubilized proteoglycans in Schwann cells. *J. Cell Biol.*, 108:1891-1897.

Carlone, R.L., Ganagarajah, M., and Rathbone, M.P. (1981): Bovine pituitary fibroblast growth factor has neurotrophic activity on newt limb regenerates and skeletal muscles in vitro. *Exp. Cell Res.*, 132:15-21.

Casscells, W., Speir, E., Sasse, J., Klagsbrun, M., Allen, P., Lee, M., Calvo, B., Chiba, M., Haggroth, L., Folkman, J., and et-al, (1990): Isolation, characterization, and localization of heparin-binding growth factors in the heart. *J. Clin. Invest.*, 85:433-441.

Castellot, J.J.J., Cochran, D.L., and Karnovsky, M.J. (1985): Effect of heparin on vascular smooth muscle cells. I. Cell metabolism. *J. Cell Physiol.*, 124:21-28.

Chambard, J.C., Paris, S., L'Allemain, G., and Pouyssegur, J. (1987): Two growth factor signalling pathways in fibroblasts distinguished by pertussis toxin. *Nature*, 326:800-803.

Champion-Arnaud, P., Ronsin, C., Gilbert, E., Gesnel, M.C., Houssaint, E., and Breathnach, R. (1991): Multiple mRNAs code for proteins related to the BEK fibroblast growth factor receptor. *Oncogene*, 6:979-987.



Chandrasekaran, E.V. and BeMiller, J.N. (1980): Constituent analysis of glycosaminoglycans. In: *Methods in carbohydrate chemistry*, edited by R.L. Whistler, et al, pp. 89-96. Academic Press,

Chaproniere, D.M. and McKeehan, W.L. (1986): Serial culture of single adult human prostatic epithelial cells in serum-free medium containing low calcium and a new growth factor from bovine brain. *Cancer Res.*, 46:819-824.

Chou, P.Y. and Fasman, G.D. (1978): Empirical predictions of protein conformation. *Annu.Rev.Biochem.*, 47:251-276.

Church, F.C. and Griffith, M.J. (1984): Evidence for essential lysines in heparin cofactor II. *Biochem.Biophys.Res.Comm.*, 124:745-751.

Clegg, C.H., Linkhart, T.A., Olwin, B.B., and Hauschka, S.D. (1987): Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. *J.Cell Biol.*, 105:949-956.

Cooper, D.N. and Barondes, S.H. (1990): Evidence for export of a muscle lectin from cytosol to extracellular matrix and for a novel secretory mechanism. *J.Cell Biol.*, 110:1681-1691.

Copeland, R.A., Ji, H., Halfpenny, A.J., Williams, R.W., Thompson, K.C., Herber, W.K., Thomas, K.A., Bruner, M.W., Ryan, J.A., Marquis-Omer, D., and et-al, (1991): The structure of human acidic fibroblast growth factor and its interaction with heparin. *Arch.Biochem.Biophys.*, 289:53-61.

Cordon-Cardo, C., Vlodavsky, I., Haimovitz-Friedman, A., Hicklin, D., and Fuks, Z. (1990): Expression of basic fibroblast growth factor in normal human tissues. *Lab.Invest.*, 63:832-840.

Coughlin, S.R., Barr, P.J., Cousens, L.S., Fretto, L.J., and Williams, L.T. (1988): Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity in vivo. *J.Biol.Chem.*, 263:988-993.

Courty, J., Loret, C., Moenner, M., Chevallier, B., Lagente, O., Courtois, Y., and Barritault, D. (1985): Bovine retina contains three growth factor activities with different affinity to heparin: eye derived growth factor I, II, III. *Biochimie*, 67:265-269.

Courty, J., Chevallier, B., Moenner, M., Loret, C., Lagente, O., Bohlen, P., Courtois, Y., and Barritault, D. (1986): Evidence for FGF-like growth factor in adult bovine retina: analogies with EDGF I. *Biochem.Biophys.Res.Comm.*, 136:102-108.

Courty, J., Dauchel, M.C., Mereau, A., Badet, J., and Barritault, D. (1988): Presence of basic fibroblast growth factor receptors in bovine brain membranes. *J.Biol.Chem.*, 263:11217-11220.



- Crabb, J.W., Armes, L.G., Carr, S.A., Johnson, C.M., Roberts, G.D., Bordoli, R.S., and McKeehan, W.L. (1986): Complete primary structure of prostatropin, a prostate epithelial cell growth factor. *Biochemistry*, 25:4988-4993.
- Crumley, G., Bellot, F., Kaplow, J.M., Schlessinger, J., Jaye, M., and Dionne, C.A. (1991): High-affinity binding and activation of a truncated FGF receptor by both aFGF and bFGF. *Oncogene*, 6:2255-2262.
- Cuevas, P., Carceller, F., Esteban, A., Baird, A., and Guillemin, R. (1988): Basic fibroblast growth factor (bFGF) enhances retinal ganglion cell survival and promotes axonal growth of rat transected optic nerve. *Third Joint Meeting on Neurochemical approaches to the understanding of cerebral disorders, European Society for Neurochemistry*, (Abstract)
- D'Amore, P.A. and Klagsbrun, M. (1984): Endothelial cell mitogens derived from retina and hypothalamus: biochemical and biological similarities. *J. Cell Biol.*, 99:1545-1549.
- D'Amore, P.A. (1990a): Modes of FGF release in vivo and in vitro. *Cancer Metastasis Rev.*, 9:227-238.
- D'Amore, P.A. (1990b): Heparin-endothelial cell interactions. *Haemostasis*, 20(suppl.1):159-165.
- Damon, D.H., D'Amore, P.A., and Wagner, J.A. (1988): Sulfated glycosaminoglycans modify growth factor-induced neurite outgrowth in PC12 cells. *J. Cell. Physiol.*, 135:293-300.
- Damon, D.H., Lobb, R.R., D'Amore, P.A., and Wagner, J.A. (1989): Heparin potentiates the action of acidic fibroblast growth factor by prolonging its biological half-life. *J. Cell Physiol.*, 138:221-226.
- Dell'Era, P., Presta, M., and Ragnotti, G. (1991): Nuclear localization of endogenous basic fibroblast growth factor in cultured endothelial cells. *Exp. Cell Res.*, 192:505-510.
- Delli-Bovi, P., Curatola, A.M., Newman, K.M., Sato, Y., Moscatelli, D., Hewick, R.M., Rifkin, D.B., and Basilico, C. (1988): Processing, secretion, and biological properties of a novel growth factor of the fibroblast growth factor family with oncogenic potential. *Mol. Cell Biol.*, 8:2933-2941.
- Deuel, T.F., Keim, P.S., Farmer, M., and Henrikson, R.L. (1977): Amino acid sequence of human platelet factor 4. *Proc. Natl. Acad. Sci. U.S.A.*, 74:2256-2258.
- Deuel, T.F., Senior, R.M., Chang, D., Griffin, G.L., Henrikson, R.L., and Kaiser, E.T. (1981): Platelet factor 4 is chemotactic for neutrophils and monocytes. *Proc. Natl. Acad. Sci. U.S.A.*, 78:4584-4587.
- Dickson, C., Smith, R., Brookes, S., and Peters, G. (1984): Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region int-2. *Cell*, 37:529-536.



Dickson, C. and Peters, G. (1987): Potential oncogene product related to growth factors [letter]. *Nature*, 326:833

Dionne, C.A., Crumley, G., Bellot, F., Kaplow, J.M., Searfoss, G., Ruta, M., Burgess, W.H., Jaye, M., and Schlessinger, J. (1990): Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. *EMBO J.*, 9:2685-2692.

Duan, D.S., Werner, S., and Williams, L.T. (1992): A naturally occurring secreted form of fibroblast growth factor (FGF) receptor 1 binds basic FGF in preference over acidic FGF. *J.Biol.Chem.*, 267:16076-16080.

Eccleston, P.A. and Silberberg, D.H. (1985): Fibroblast growth factor is a mitogen for oligodendrocytes in vitro. *Brain Res.*, 353:315-318.

Eisemann, A., Ahn, J.A., Graziani, G., Tronick, S.R., and Ron, D. (1991): Alternative splicing generates at least five different isoforms of the human basic-FGF receptor [published erratum appears in *Oncogene* 1991 Dec;6(12):2379]. *Oncogene*, 6:1195-1202.

Elliott, B., Ostman, A., Westermarck, B., and Rubin, K. (1992): Modulation of growth factor responsiveness of murine mammary carcinoma cells by cell matrix interactions: correlation of cell proliferation and spreading. *J.Cell Physiol.*, 152:292-301.

Engleka, K.A. and Maciag, T. (1992): Inactivation of human fibroblast growth factor-1 (FGF-1) activity by interaction with copper ions involves FGF-1 dimer formation induced by copper-catalyzed oxidation. *J.Biol.Chem.*, 267:11307-11315.

Eriksson, A.E., Cousens, L.S., Weaver, L.H., and Matthews, B.W. (1991): Three-dimensional structure of human basic fibroblast growth factor. *Proc.Natl.Acad.Sci.U.S.A.*, 88:3441-3445.

Esch, F., Baird, A., Ling, N., Ueno, N., Hill, F., Denoroy, L., Klepper, R., Gospodarowicz, D., Bohlen, P., and Guillemin, R. (1985): Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF. *Proc.Natl.Acad.Sci.U.S.A.*, 82:6507-6511.

Fager, G., Camejo, G., Olsson, U., Ostergren-Lunden, G., and Bondjers, G. (1992): Heparin-like glycosaminoglycans influence growth and phenotype of human arterial smooth muscle cells in vitro. II. The platelet-derived growth factor A-chain contains a sequence that specifically binds heparin. *In Vitro Cell Dev.Biol.*, 28A:176-180.

Fedarko, N.S. and Conrad, H.E. (1986): A unique heparan sulfate in the nuclei of hepatocytes: structural changes with the growth state of the cells. *J.Cell Biol.*, 102:587-599.



- Feige, J.J. and Baird, A. (1989): Basic fibroblast growth factor is a substrate for protein phosphorylation and is phosphorylated by capillary endothelial cells in culture. *Proc.Natl.Acad.Sci.U.S.A*, 86:3174-3178.
- Ferrara, N., Schweigerer, L., Neufeld, G., Mitchell, R., and Gospodarowicz, D. (1987): Pituitary follicular cells produce basic fibroblast growth factor. *Proc.Natl.Acad.Sci.U.S.A*, 84:5773-5777.
- Files, J.C., Malpass, T.W., Yee, E.K., Ritchie, J.L., and Harker, L.A. (1981): Studies of human plate alpha-granule release in vivo. *Blood*, 58:607-618.
- Finch, P.W., Rubin, J.S., Miki, T., Ron, D., and Aaronson, S.A. (1989): Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science*, 245:752-755.
- Fitzpatrick, L.R., Jakubowska, A., Martin, G.E., Davis, M., Jaye, M.C., and Dionne, C.A. (1992): aFGF accelerates the healing of acetic-acid-induced gastric ulcers in rats. *Digestion*, 53:17-27.
- Flaumenhaft, R., Moscatelli, D., Saksela, O., and Rifkin, D.B. (1989): Role of extracellular matrix in the action of basic fibroblast growth factor: matrix as a source of growth for long-term stimulation of plasminogen activator production and DNA synthesis. *J.Cell.Physiol.*, 140:75-81.
- Florkiewicz, R.Z. and Sommer, A. (1989): Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons [published erratum appears in Proc Natl Acad Sci U S A 1990 Mar; 87(5):2045]. *Proc.Natl.Acad.Sci.U.S.A*, 86:3978-3981.
- Florkiewicz, R.Z., Shibata, F., Barankiewicz, T., Baird, A., Gonzalez, A.M., Florkiewicz, E., and Shah, N. (1991a): Basic fibroblast growth factor gene expression. *Ann.N.Y.Acad.Sci.*, 638:109-126.
- Florkiewicz, R.Z., Baird, A., and Gonzalez, A.M. (1991b): Multiple forms of bFGF: differential nuclear and cell surface localization. *Growth Factors*, 4:265-275.
- Folkman, J. (1987): *Xlth Congress of Thrombosis and Haemostasis*. Leuven University Press, Leuven.
- Folkman, J. (1990): What is the evidence that tumors are angiogenesis dependent? [editorial]. *J.Natl.Cancer Inst.*, 82:4-6.
- Folkman, J. (1991): *Biologic Therapy of Cancer*. Lippincott Co., Philadelphia.
- Folkman, J. and Moscona, A. (1978): Role of cell shape in growth control. *Nature*, 273:345-349.
- Folkman, J. and Klagsburn, M. (1987): Angiogenic factors. *Science*, 235:442-447.



Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D., and Vlodavsky, I. (1988): A heparin-binding angiogenic protein--basic fibroblast growth factor--is stored within basement membrane. *Am.J.Pathol.*, 130:393-400.

Folkman, J., Szabo, S., Vattay, P., Morales, R., Pinkus, G., and Kato, K. (1990): Effect of orally administered bFGF on healing of chronic duodenal ulcers, gastric secretion, and acute mucosal lesions in rats. *Gastroenterology*, 98:A45:

Folkman, J., Szabo, S., Stovroff, M., McNeil, P., Li, W., and Shing, Y. (1991): Duodenal ulcer. Discovery of a new mechanism and development of angiogenic therapy that accelerates healing. *Ann.Surg.*, 214:414-25; discus.

Folkman, J. and Brem, H. (1992): Angiogenesis and Inflammation. In: *Inflammation: Basic Principles and Clinical Correlates*, edited by I.M. Goldstein, et al, pp. 821-839. Raven Press, NY.

Folkman, J. and Shing, Y. (1992): Angiogenesis. *J.Biol.Chem.*, 267:10931-10934.

Fox, G.M., Schiffer, S.G., Rohde, M.F., Tsai, L.B., Banks, A.R., and Arakawa, T. (1988): Production, biological activity, and structure of recombinant basic fibroblast growth factor and an analog with cysteine replaced by serine. *J.Biol.Chem.*, 263:18452-18458.

Fransson, L-A. (1987): Structure and function of cell-associated proteoglycans. *TIBS*, 12:406-411.

Friesel, R., Burgess, W.H., Mehlman, T., and Maciag, T. (1986): The characterization of the receptor for endothelial cell growth factor by covalent ligand attachment. *J.Biol.Chem.*, 261:7581-7584.

Friesel, R. and Maciag, T. (1988): Internalization and degradation of heparin binding growth factor-I by endothelial cells. *Biochem.Biophys.Res.Comm.*, 151:957-964.

Friesel, R., Burgess, W.H., and Maciag, T. (1989): Heparin-binding growth factor 1 stimulates tyrosine phosphorylation in NIH 3T3 cells. *Mol.Cell Biol.*, 9:1857-1865.

Gallagher, J.T. and Walker, A. (1985): Molecular distinctions between heparan sulphate and heparin. Analysis of sulphation patterns indicates that heparan sulphate and heparin are separate families of N-sulphated polysaccharides. *Biochem.J.*, 230:665-674.

Gallagher, J.T., Lyon, M., and Steward, W.P. (1986): Structure and function of heparan sulphate proteoglycans. *Biochem.J.*, 236:313-325.

Gallagher, J.T., Walker, A., Lyon, M., and Evans, W.H. (1988): Heparan sulphate-degrading endoglycosidase in liver plasma membranes. *Biochem.J.*, 250:719-726.



- Gallagher, J.T. and Turnbull, J.E. (1992): Heparan sulphate in the binding and activation of basic fibroblast growth factor. *Glycobiology*, 2:523-528.
- Gimenez-Gallego, G., Rodkey, J., Bennett, C., Rios-Candelore, M., DiSalvo, J., and Thomas, K. (1985): Brain-derived acidic fibroblast growth factor: complete amino acid sequence and homologies. *Science*, 230:1385-1388.
- Gimenez-Gallego, G., Conn, G., Hatcher, V.B., and Thomas, K.A. (1986): Human brain-derived acidic and basic fibroblast growth factors: amino terminal sequences and specific mitogenic activities. *Biochem.Biophys.Res.Comm.*, 135:541-548.
- Gitay-Goren, H., Soker, S., Vlodavsky, I., and Neufeld, G. (1992): The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. *J.Biol.Chem.*, 267:6093-6098.
- Givol, D. and Yayon, A. (1992): Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *FASEB J.*, 6:3362-3369.
- Glabe, C.G., Harty, P.K., and Rosen, S.D. (1983): Preparation and properties of fluorescent polysaccharides. *Anal.Biochem.*, 130:287-294.
- Glazer, L. and Shilo, B.Z. (1991): The Drosophila FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes Dev.*, 5:697-705.
- Goldschmidt-Clermont, P.J., Kim, J.W., Machesky, L.M., Rhee, S.G., and Pollard, T.D. (1991): Regulation of phospholipase C-gamma 1 by profilin and tyrosine phosphorylation. *Science*, 251:1231-1233.
- Gonzalez, A.M., Buscaglia, M., Ong, M., and Baird, A. (1990): Distribution of basic fibroblast growth factor in the 18-day rat fetus: localization in the basement membranes of diverse tissues. *J.Cell Biol.*, 110:753-765.
- Goodwin, R.G., Friend, D., Ziegler, S.F., Jerzy, R., Falk, B.A., Gimpel, S., Cosman, D., Dower, S.K., March, C.J., Namen, A.E., and et-al, (1990): Cloning of the human and murine interleukin-7 receptors: demonstration of a soluble form and homology to a new receptor superfamily. *Cell*, 60:941-951.
- Gordon, M.Y., Riley, G.P., Watt, S.M., and Greaves, M.F. (1987): Compartmentalization of a haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. *Nature*, 326:403-405.
- Gospodarowicz, D. (1975): Purification of a fibroblast growth factor from bovine pituitary. *J.Biol.Chem.*, 250:2515-2520.
- Gospodarowicz, D. (1983): Growth factors and their action in vivo and in vitro. *J.Pathol.*, 141:201-233.



Gospodarowicz, D. (1987): Isolation and characterization of acidic and basic fibroblast growth factor. *Methods Enzymol.*, 147:106-119.

Gospodarowicz, D. (1990): Fibroblast growth factor. Chemical structure and biologic function. *Clin.Orthop.*, 231-248.

Gospodarowicz, D. and Mescher, A.L. (1977): A comparison of the responses of cultured myoblasts and chondrocytes to fibroblast and epidermal growth factors. *J.Cell Physiol.*, 93:117-127.

Gospodarowicz, D. and Bialecki, H. (1978): The effects of the epidermal and fibroblast growth factors on the replicative lifespan of cultured bovine granulosa cells. *Endocrinology*, 103:854-865.

Gospodarowicz, D. and Cheng, J. (1986): Heparin protects basic and acidic FGF from inactivation. *J.Cell Physiol.*, 128:475-484.

Gospodarowicz, D., Jones, K.L., and Sato, G. (1974): Purification of a growth factor for ovarian cells from bovine pituitary glands. *PNAS*, 71:2295-2299.

Gospodarowicz, D., Rudland, P., Lindstrom, J., and Benirschke, K. (1975): Fibroblast growth factor: its localization, purification, mode of action, and physiological significance. *Adv.Metab.Disord.*, 8:301-335.

Gospodarowicz, D., Weseman, J., Moran, J.S., and Lindstrom, J. (1976): Effect of fibroblast growth factor on the division and fusion of bovine myoblasts. *J.Cell Biol.*, 70:395-405.

Gospodarowicz, D., Ill, C.R., Hornsby, P.J., and Gill, G.N. (1977): Control of bovine adrenal cortical cell proliferation by fibroblast growth factor. Lack of effect of epidermal growth factor. *Endocrinology*, 100:1080-1089.

Gospodarowicz, D., Greenburg, G., Bialecki, H., and Zetter, B.R. (1978): Factors involved in the modulation of cell proliferation in vivo and in vitro: the role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. *In Vitro Cell.Dev.Biol.*, 14:85-118.

Gospodarowicz, D., Bialecki, H., and Thakral, T.K. (1979): The angiogenic activity of the fibroblast and epidermal growth factor. *Exp.Eye Res.*, 28:501-514.

Gospodarowicz, D., Cheng, J., Lui, G.M., Baird, A., and Bohlent, P. (1984): Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: identity with pituitary fibroblast growth factor. *Proc.Natl.Acad.Sci.U.S.A.*, 81:6963-6967.

Gospodarowicz, D., Cheng, J., Lui, G.M., Baird, A., Esch, F., and Bohlen, P. (1985a): Corpus luteum angiogenic factor is related to fibroblast growth factor. *Endocrinology*, 117:2383-2391.



- Gospodarowicz, D., Cheng, J., Lui, G.M., Fujii, D.K., Baird, A., and Bohlen, P. (1985b): Fibroblast growth factor in the human placenta. *Biochem.Biophys.Res.Comm.*, 128:554-562.
- Gospodarowicz, D., Baird, A., Cheng, J., Lui, G.M., Esch, F., and Bohlen, P. (1986a): Isolation of fibroblast growth factor from bovine adrenal gland: physicochemical and biological characterization. *Endocrinology*, 118:82-90.
- Gospodarowicz, D., Neufeld, G., and Schweigerer, L. (1986b): Molecular and biological characterization of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. *Cell Differ.*, 19:1-17.
- Gospodarowicz, D., Neufeld, G., and Schweigerer, L. (1986c): Fibroblast growth factor. *Mol.Cell Endocrinol.*, 46:187-204.
- Gospodarowicz, D., Ferrara, N., Schweigerer, L., and Neufeld, G. (1987a): Structural characterization and biological functions of fibroblast growth factor. *Endocr.Rev.*, 8:95-114.
- Gospodarowicz, D., Neufeld, G., and Schweigerer, L. (1987b): Fibroblast growth factor: structural and biological properties. *J.Cell Physiol.Suppl.*, Suppl 5:15-26.
- Greenfield, C., Hiles, I., Waterfield, M.D., Federwisch, M., Wollmer, A., Blundell, T.L., and McDonald, N. (1989): Epidermal growth factor binding induces a conformational change in the external domain of its receptor. *EMBO J.*, 8:4115-4123.
- Grunz, H., McKeehan, W.L., Knochel, W., Born, J., and Tiedemann, H. (1988): Induction of mesodermal tissues by acidic and basic heparin binding growth factors. *Cell Differ.*, 22:183-189.
- Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K., and Kimata, K. (1992): Structure of a heparan sulphate oligosaccharide that binds to basic fibroblast growth factor. *Biochem.J.*, 285:805-813.
- Hajjar, D.P., Boyd, D.B., Harpel, P.C., and Nachman, R.L. (1987): Histidine-rich glycoprotein inhibits the antiproliferative effect of heparin on smooth muscle cells. *J.Exp.Med.*, 165:908-913.
- Halaban, R., Ghosh, S., and Baird, A. (1987): bFGF is the putative natural growth factor for human melanocytes. *In Vitro Cell Dev.Biol.*, 23:47-52.
- Halperin, J.A. and Lobb, R.R. (1987): Effect of heparin-binding growth factors on monovalent cation transport in Balb/C 3T3 cells. *Biochem.Biophys.Res.Comm.*, 144:115-122.
- Hammacher, A., Mellstrom, K., Heldin, C.H., and Westermark, B. (1989): Isoform-specific induction of actin reorganization by platelet-derived growth factor suggests that the functionally active receptor is a dimer. *EMBO J.*, 8:2489-2495.



Hansson, H.A. and Norstrom, E. (1991): aFGF and EGF and their receptors in the normal and healing rat gastric mucosa. *Digestion*, 49(suppl 1):17-18.(Abstract)

Harper, J.W. and Lobb, R.R. (1988): Reductive methylation of lysine residues in acidic fibroblast growth factor: effect on mitogenic activity and heparin affinity. *Biochemistry*, 27:671-678.

Hattori, Y., Odagiri, H., Nakatani, H., Miyagawa, K., Naito, K., Sakamoto, H., Katoh, O., Yoshida, T., Sugimura, T., and Terada, M. (1990): K-sam, an amplified gene in stomach cancer, is a member of the heparin-binding growth factor receptor genes. *Proc.Natl.Acad.Sci.U.S.A.*, 87:5983-5987.

Haub, O., Drucker, B., and Goldfarb, M. (1990): Expression of the murine fibroblast growth factor 5 gene in the adult central nervous system. *Proc.Natl.Acad.Sci.U.S.A.*, 87:8022-8026.

Haub, O. and Goldfarb, M. (1991): Expression of the fibroblast growth factor-5 gene in the mouse embryo. *Development*, 112:397-406.

Haupt, H. and Heimbürger, N. (1972): [Human serum proteins with high affinity for carboxymethylcellulose. I. Isolation of lysozyme, C1q and 2 hitherto unknown -globulins]. *Hoppe Seylers.Z.Physiol.Chem.*, 353:1125-1132.

Hauschka, P.V., Mavrakos, A.E., Iafrafi, M.D., Doleman, S.E., and Klagsbrun, M. (1986): Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-Sepharose. *J.Biol.Chem.*, 261:12665-12674.

Hay, E.D. (1974): Cellular Basis of Regeneration. In: *Concepts in Development*, edited by J. Lash, et al, pp. 404-428. Sinauer Stanford Com.,

Hay, N., Takimoto, M., and Bishop, J.M. (1989): A FOS protein is present in a complex that binds a negative regulator of MYC. *Genes Dev.*, 3:293-303.

Hayashi, I., Larner, J., and Sato, G. (1978): Hormonal growth control of cells in culture. *In Vitro Cell.Dev.Biol.*, 14:23-30.

Heimbürger, N., Haupt, H., Kranz, T., and Baudner, S. (1972): [Human serum proteins with high affinity to carboxymethylcellulose. II. Physico-chemical and immunological characterization of a histidine-rich 3,8S- 2 -glycoprotein (CM-protein I)]. *Hoppe Seylers.Z.Physiol.Chem.*, 353:1133-1140.

Heldin, C.H., Ernlund, A., Rorsman, C., and Ronnstrand, L. (1989): Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. *J.Biol.Chem.*, 264:8905-8912.

Herbert, J.M., Basilico, C., Goldfarb, M., Haub, O., and Martin, G.R. (1990): Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression patterns during embryogenesis. *Dev.Biol.*, 138:454-463.



- Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C., and Klagsbrun, M. (1991): A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science*, 936:939.
- Hill, D.J. and Logan, A. (1992): Cell cycle-dependent localization of immunoreactive basic fibroblast growth factor to cytoplasm and nucleus of isolated ovine fetal growth plate chondrocytes. *Growth Factors*, 7:215-231.
- Hoffman, A.S. (1940): The growth-activating effect of extracts of adult and embryonic tissues of rat on fibroblast colonies in vitro. *Growth*, 4:361-376.
- Holmer, E., Soderstrom, G., and Andersson, L.O. (1979): Studies on the mechanism of the rate-enhancing effect of heparin on the thrombin-antithrombin III reaction. *Eur.J.Biochem.*, 93:1-5.
- Hook, M., Kjellen, L., and Johansson, S. (1984): Cell-surface glycosaminoglycans. *Annu.Rev.Biochem.*, 53:847-869.
- Horner, A.A. (1971): Macromolecular heparin from rat skin. Isolation, characterization, and depolymerization with ascorbate. *J.Biol.Chem.*, 246:231-239.
- Horner, A.A. (1977): The concept of macromolecular heparin and its physiological significance. *Fed.Proc.*, 36:35-39.
- Horton, J.E., Harper, J., and Harper, E. (1980): Platelet factor 4 regulates osteoclastic bone resorption in vitro. *Biochim.Biophys.Acta*, 630:459-462.
- Hoshi, H. and McKeegan, W.L. (1984): Brain- and liver cell-derived factors are required for growth of human endothelial cells in serum-free culture. *Proc.Natl.Acad.Sci.U.S.A*, 81:6413-6417.
- Hou, J., Kan, M., McKeegan, K., McBride, G., Adams, P., and McKeegan, W. (1991): Fibroblast growth factor receptors from liver vary in three structural domains. *Science*, 251:665-668.
- Houssaint, E., Blanquet, P.R., Champion-Arnaud, P., Gesnel, M.C., Torriglia, A., Courtois, Y., and Breathnach, R. (1990): Related fibroblast growth factor receptor genes exist in the human genome. *Proc.Natl.Acad.Sci.U.S.A*, 87:8180-8184.
- Huang, S.S., Huang, J.S. (1986): Association of bovine brain-derived growth factor receptor with protein tyrosine kinase activity. *J.Biol.Chem.*, 261:9568-9571
- Huang, J.S., Huang, S.S., and Kuo, M.D. (1986): Bovine brain-derived growth factor. Purification and characterization of its interaction with responsive cells. *J.Biol.Chem.*, 261:11600-11607.



- Huang, S.S., Tsai, C.C., Adams, S.P., and Huang, J.S. (1987): Neuron localization and neuroblastoma cell expression of brain-derived growth factor. *Biochem. Biophys. Res. Commun.*, 144:81-87.
- Humphries, M.J., Akiyama, S.K., Komoriya, A., Olden, K., and Yamada, K.M. (1986): Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion. *J. Cell Biol.*, 103:2637-2647.
- Hynes, R.O. (1987): Integrins: A family of cell surface receptors. *Cell*, 48:549-554.
- Ichinose, A., Mimuro, J., Koide, T., and Aoki, N. (1984): Histidine-rich glycoprotein and alpha 2-plasmin inhibitor in inhibition of plasminogen binding to fibrin. *Thromb. Res.*, 33:401-407.
- Imamura, T., Engleka, K., Zhan, X., Tokita, Y., Forough, R., Roeder, D., Jackson, A., Maier, J.A., Hla, T., and Maciag, T. (1990): Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence. *Science*, 249:1567-1570.
- Ingber, D.E. (1990a): Fibronectin controls capillary endothelial cell growth by modulating cell shape. *Proc. Natl. Acad. Sci. U.S.A.*, 87:3579-3583.
- Ingber, D.E. (1990b): Control of fibroblast growth factor action by extracellular matrix. In: *Growth factors in health and disease*, edited by B. Westermark, et al, pp. 289-302. Elsevier Sci.Pub., Amsterdam.
- Inoue, K., Sakai, T., and Hattori, M. (1991): The cell-adhesive effect of basic fibroblast growth factor on pituitary cells in vitro. *J. Endocrinol.*, 130:381-386.
- Ishai-Michaeli, R., Eldor, A., and Vlodavsky, I. (1990): Heparanase activity expressed by platelets, neutrophils, and lymphoma cells releases active fibroblast growth factor from extracellular matrix. *Cell Regul.*, 1:833-842.
- Ishihara, M., Fedarko, N.S., and Conrad, H.E. (1987): Involvement of phosphatidylinositol and insulin in the coordinate regulation of proteoglycan sulfate metabolism and hepatocyte growth. *J. Biol. Chem.*, 262:4708-4716.
- Ishihara, M., Tyrrell, D.J., Kiefer, M.C., Barr, P.J., and Swiedler, S.J. (1992): A cell-based assay for evaluating the interaction of heparin-like molecules and basic fibroblast growth factor. *Anal. Biochem.*, 202:310-315.
- Jackson, R.L., Busch, S.J., and Cardin, A.D. (1991): Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. *Physiol. Rev.*, 71:481-539.
- Jaffe, E.A. (1984): *Biology of Endothelial Cells*. Martinus-Nijhoff, The Hague.
- Jakobovits, A., Shackleford, G.M., Varmus, H.E., and Martin, G.R. (1986): Two proto-oncogenes implicated in mammary carcinogenesis, int-1 and int-2, are independently regulated during mouse development. *Proc. Natl. Acad. Sci. U.S.A.*, 83:7806-7810.



- Jansson, L., Ogren, S., and Lindahl, U. (1975): Macromolecular properties and end-group analysis of heparin isolated from bovine liver capsule. *Biochem.J.*, 145:53-62.
- Jaye, M., Howk, R., Burgess, W., Ricca, G.A., Chiu, I.M., Ravera, M.W., O'Brien, S.J., Modi, W.S., Maciag, T., and Drohan, W.N. (1986): Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosome localization. *Science*, 233:541-545.
- Jaye, M., Lyall, R.M., Mudd, R., Schlessinger, J., and Sarver, N. (1988): Expression of acidic fibroblast growth factor cDNA confers growth advantage and tumorigenesis to Swiss 3T3 cells. *EMBO J.*, 7:963-969.
- Jeanny, J.C., Fayein, N., Moenner, M., Chevallier, B., Barritault, D., and Courtois, Y. (1987): Specific fixation of bovine brain and retinal acidic and basic fibroblast growth factors to mouse embryonic eye basement membranes. *Exp.Cell Res.*, 171:63-75.
- Johnson, D.E., Lee, P.L., Lu, J., and Williams, L.T. (1990): Diverse forms of a receptor for acidic and basic fibroblast growth factors. *Mol.Cell Biol.*, 10:4728-4736.
- Johnson, L.N. (1991): Direct interaction or remote control? *Current Biology*, 1:17-19.
- Johnson, D.E. and Williams, L.T. (1993): Structural and functional diversity in the FGF receptor multigene family. *Adv.Cancer Res.*, 60:1-41.
- Joseph-Silverstein, J., Consigli, S.A., Lyser, K.M., and Ver-Pault, C. (1989): Basic fibroblast growth factor in the chick embryo: immunolocalization to striated muscle cells and their precursors. *J.Cell Biol.*, 108:2459-2466.
- Kan, M., DiSorbo, D., Hou, J.Z., Hoshi, H., Mansson, P.E., and McKeehan, W.L. (1988): High and low affinity binding of heparin-binding growth factor to a 130-kDa receptor correlates with stimulation and inhibition of growth of a differentiated human hepatoma cell. *J.Biol.Chem.*, 263:11306-11313.
- Kan, M., Wang, F., Xu, J., Crabb, J.W., Hou, J., and McKeehan, W.L. (1993): An essential heparin-binding domain in the fibroblast growth factor receptor kinase. *Science*, 259:1918-1921.
- Kaplow, J.M., Bellot, F., Crumley, G., Dionne, C.A., and Jaye, M. (1990): Effect of heparin on the binding affinity of acidic FGF for the cloned human FGF receptors, flg and bek. *Biochem.Biophys.Res.Comm.*, 172:107-112.
- Kardami, E. and Fandrich, R.R. (1989): Basic fibroblast growth factor in atria and ventricles of the vertebrate heart. *J.Cell Biol.*, 109:1865-1875.
- Kardami, E., Spector, D., and Strohman, R.C. (1985): Myogenic growth factor present in skeletal muscle is purified by heparin-affinity chromatography. *Proc.Natl.Acad.Sci.U.S.A.*, 82:8044-8047.



Kato, Y. and Gospodarowicz, D. (1985): Sulfated proteoglycan synthesis by confluent cultures of rabbit costal chondrocytes grown in the presence of fibroblast growth factor. *J.Cell Biol.*, 100:477-485.

Kato, Y., Iwamoto, M., and Koike, T. (1987): Fibroblast growth factor stimulates colony formation of differentiated chondrocytes in soft agar. *J.Cell Physiol.*, 133:491-498.

Katz, I.R., Thorbecke, G.J., Bell, M.K., Yin, J.Z., Clarke, D., and Zucker, M.B. (1986): Protease-induced immunoregulatory activity of platelet factor 4 [published erratum appears in *Proc Natl Acad Sci U S A* 1986 Sep; 83(17):6664]. *Proc.Natl.Acad.Sci.U.S.A*, 83:3491-3495.

Keating, A. and Gordon, M.Y. (1988): Hierarchical organization of hematopoietic microenvironments: role of proteoglycans. *Leukemia*, 2:766-769.

Keegan, K., Johnson, D.E., Williams, L.T., and Hayman, M.J. (1991): Isolation of an additional member of the fibroblast growth factor receptor family, FGFR-3. *Proc.Natl.Acad.Sci.U.S.A*, 88:1095-1099.

Kessler, D.A., Langer, R.S., Pless, N.A., and Folkman, J. (1976): Mast cells and tumor angiogenesis. *Int.J.Cancer*, 18:703-709.

Kiefer, M.C., Stephans, J.C., Crawford, K., Okino, K., and Barr, P.J. (1990): Ligand-affinity cloning and structure of a cell surface heparan sulfate proteoglycan that binds basic fibroblast growth factor. *Proc.Natl.Acad.Sci.U.S.A*, 87:6985-6989.

Kimelman, D., Abraham, J.A., Haaparanta, T., Palisi, T.M., and Kirschner, M.W. (1988): The presence of fibroblast growth factor in the frog egg: its role as a natural mesoderm inducer. *Science*, 242:1053-1056.

Kjellen, L. and Lindahl, U. (1991): Proteoglycans: structures and interactions [published erratum appears in *Annu Rev Biochem* 1992;61:following viii]. *Annu.Rev.Biochem.*, 60:443-475.

Klagsbrun, M. and Shing, Y. (1985): Heparin affinity of anionic and cationic capillary endothelial cell growth factors: analysis of hypothalamus-derived growth factors and fibroblast growth factors. *Proc.Natl.Acad.Sci.U.S.A*, 82:805-809.

Klagsbrun, M. and Baird, A. (1991): A dual receptor system is required for basic fibroblast growth factor activity. *Cell*, 67:229-231.

Knochel, W., Born, J., Hoppe, P., Loppnow-Blinde, B., Tiedemann, H., McKeehan, W.L., and Grunz, H. (1987): Mesoderm-inducing factors. Their possible relationship to heparin-binding growth factors and transforming growth factor-beta. *Naturwissenschaften.*, 74:604-606.



- Koide, T., Odani, S., and Ono, T. (1985): Human histidine-rich glycoprotein: simultaneous purification with antithrombin III and characterization of its gross structure. *J.Biochem.Tokyo.*, 98:1191-1200.
- Kornbluth, S., Paulson, K.E., and Hanafusa, H. (1988): Novel tyrosine kinase identified by phosphotyrosine antibody screening of cDNA libraries. *Mol.Cell Biol.*, 8:5541-5544.
- Kowalski, S. and Finlay, T.H. (1979): Heparin and the inactivation of thrombin by antithrombin III. *Thromb.Res.*, 14:387-397.
- Kurokawa, T., Sasada, R., Iwane, M., and Igarashi, K. (1987): Cloning and expression of cDNA encoding human basic fibroblast growth factor. *FEBS Lett.*, 213:189-194.
- Kurokawa, M., Doctrow, S.R., and Klagsbrun, M. (1989): Neutralizing antibodies inhibit the binding of basic fibroblast growth factor to its receptor but not to heparin. *J.Biol.Chem.*, 264:7686-7691.
- Lane, D.A., Ireland, H., Wolff, S., Ranasinghe, E., and Dawes, J. (1984): Detection of enhanced in vivo platelet alpha-granule release in different patient groups--comparison of beta-thromboglobulin, platelet factor 4 and thrombospondin assays. *Thromb.Haemost.*, 52:183-187.
- Lane, D.A., Pejler, G., Flynn, A.M., Thompson, E.A., and Lindahl, U. (1986): Neutralization of heparin-related saccharides by histidine-rich glycoprotein and platelet factor 4 [published erratum appears in J Biol Chem 1986 Oct 5;261(28):13387]. *J.Biol.Chem.*, 261:3980-3986.
- Lee, M.K. and Lander, A.D. (1991): Analysis of affinity and structural selectivity in the binding of proteins to glycosaminoglycans: development of a sensitive electrophoretic approach. *Proc.Natl.Acad.Sci.USA*, 88:2768-2772.
- Lee, P.L., Johnson, D.E., Cousens, L.S., Fried, V.A., and Williams, L.T. (1989): Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. *Science*, 245:57-60.
- Lemmon, S.K., Riley, M.C., Thomas, K.A., Hoover, G.A., Maciag, T., and Bradshaw, R.A. (1982): Bovine fibroblast growth factor: comparison of brain and pituitary preparations. *J.Cell Biol.*, 95:162-169.
- Leung, L.L.K. (1986): Interaction of histidine-rich glycoprotein with fibrinogen and fibrin. *J.Clin.Invest.*, 77:1305-1311.
- Li, E.H., Fenton, J.W., and Feinman, R.D. (1976): The role of heparin in the thrombin-antithrombin III reaction. *Arch.Biochem.Biophys.*, 175:153-159.
- Libermann, T.A., Friesel, R., Jaye, M., Lyall, R.M., Westermarck, B., Drohan, W., Schmidt, A., Maciag, T., and Schlessinger, J. (1987): An angiogenic growth factor is expressed in human glioma cells. *EMBO J.*, 6:1627-1632.



Lijnen, H.R., Hoylaerts, M., and Collen, D. (1980): Isolation and characterization of a human plasma protein with affinity for the lysine binding sites in plasminogen. Role in the regulation of fibrinolysis and identification as histidine-rich glycoprotein. *J.Biol.Chem.*, 255:10214-10222.

Lijnen, H.R., Rylatt, D.B., and Collen, D. (1983a): Physicochemical, immunochemical and functional comparison of human histidine-rich glycoprotein and autorosette inhibition factor. *Biochim.Biophys.Acta*, 742:109-115.

Lijnen, H.R., Hoylaerts, M., and Collen, D. (1983b): Heparin binding properties of human histidine-rich glycoprotein. Mechanism and role in the neutralization of heparin in plasma. *J.Biol.Chem.*, 258:3803-3808.

Lindahl, U., Feingold, D.S., and Roden, L. (1986): Biosynthesis of heparin. *TIBS*, 11:221-225.

Lindahl, U. (1989): Biosynthesis of heparin and related polysaccharides. In: *Heparin, chemical and biological properties, clinical applications*, edited by D.A. Lane, et al, pp. 159-189. Edward Arnold, London, UK.

Linemeyer, D.L., Kelly, L.J., Menke, J.G., Gimenez-Gallego, G., DiSalvo, J., and Thomas, K.A. (1987): Expression in *Escherichia coli* of a chemically synthesized gene for biologically active bovine fibroblast growth factor. *Biotechnology*, 5:960-965.

Linemeyer, D.L., Menke, J.G., Kelly, L.J., DiSalvo, J., Soderman, D., Schaeffer, M.T., Ortega, S., Gimenez-Gallego, G., and Thomas, K.A. (1990): Disulfide bonds are neither required, present, nor compatible with full activity of human recombinant acidic fibroblast growth factor. *Growth Factors*, 3:287-298.

Lipton, S.A., Wagner, J.A., Madison, R.D., and D'Amore, P.A. (1988): Acidic fibroblast growth factor enhances regeneration of processes by postnatal mammalian retinal ganglion cells in culture. *Proc.Natl.Acad.Sci.U.S.A*, 85:2388-2392.

Lobb, R.R. and Fett, J.W. (1984): Purification of two distinct growth factors from bovine neural tissue by heparin affinity chromatography. *Biochemistry*, 23:6295-6299.

Lobb, R.R., Alderman, E.M., and Fett, J.W. (1985): Induction of angiogenesis by bovine brain derived class 1 heparin-binding growth factor. *Biochemistry*, 24:4969-4973.

Lobb, R.R., Harper, W., and Fett, J.W. (1986): Purification of heparin-binding growth factors. *Anal.Biochem.*, 154:1-14.

Lobb, R.R. (1988a): Clinical applications of heparin-binding growth factors. *Eur.J.Clin.Invest.*, 18:321-336.

Lobb, R.R. (1988b): Thrombin inactivates acidic fibroblast growth factor but not basic fibroblast growth factor. *Biochemistry*, 27:2572-2578.



Logan, A. (1990): Intracrine regulation at the nucleus--a further mechanism of growth factor activity? *J.Endocrinol.*, 125:339-343.

Logan, A. and Logan, S.D. (1991): Studies on the mechanisms of signalling and inhibition by pertussis toxin of fibroblast growth factor-stimulated mitogenesis in Balb/c 3T3 cells. *Cell Signal.*, 3:215-223.

Mach, H., Volkin, D., Burke, C.J., Middaugh, C.R., Linhardt, R.J., Fromm, J.R., and Loganathan, D. (1993): Nature of the interaction of heparin with acidic fibroblast growth factor. *Biochemistry*, 32:5480-5489.

Maciag, T. (1984): Angiogenesis. *Prog.Hemost.Thromb.*, 7:167-182.

Maciag, T., Cerundolo, J., Ilsley, S., Kelley, P.R., and Forand, R. (1979): An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. *Proc.Natl.Acad.Sci.U.S.A*, 76:5674-5678.

Maciag, T., Hoover, G.A., Stemerman, M.B., and Weinstein, R. (1981): Serial propagation of human endothelial cells in vitro. *J.Cell Biol.*, 91:420-426.

Maciag, T., Mehlman, T., Friesel, R., and Schreiber, A.B. (1984): Heparin binds endothelial cell growth factor, the principal endothelial cell mitogen in bovine brain. *Science*, 225:932-935.

Magnaldo, I., L'Allemain, G., Chambard, J.C., Moenner, M., Barritault, D., and Pouyssegur, J. (1986): The mitogenic signaling pathway of fibroblast growth factor is not mediated through polyphosphoinositide hydrolysis and protein kinase C activation in hamster fibroblasts. *J.Biol.Chem.*, 261:16916-16922.

Magnaldo, I., Pouyssegur, J., and Paris, S. (1989): Fibroblast growth factor potentiates receptor- and nonreceptor-mediated stimulation of adenylate cyclase in hamster fibroblasts. *Cell Signal.*, 1:507-517.

Maimone, M.M., Tollefsen, D.M. (1990): Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity [published erratum appears in J Biol Chem 1991 Aug 5;266(22):14830] *J.Biol.Chem.*, 265:18263-18271

Maione, T.E., Gray, G.S., Petro, J., Hunt, A.J., Donner, A.L., Bauer, S.I., Carson, H.F., and Sharpe, R.J. (1990): Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science*, 247:77-79.

Mansukhani, A., Moscatelli, D., Talarico, D., Levytska, V., and Basilico, C. (1990): A murine fibroblast growth factor (FGF) receptor expressed in CHO cells is activated by basic FGF and Kaposi FGF. *Proc.Natl.Acad.Sci.U.S.A*, 87:4378-4382.

Mansukhani, A., Dell'Era, P., Moscatelli, D., Kornbluth, S., Hanafusa, H., and Basilico, C. (1992): Characterization of the murine BEK fibroblast growth factor (FGF) receptor: activation by three members of the FGF family and requirement for heparin. *Proc.Natl.Acad.Sci.U.S.A*, 89:3305-3309.



- Marcum, J.A. and Rosenberg, R.D. (1989): The biochemistry, cell biology and pathophysiology of anticoagulant active heparin-like molecules of the vessel wall. In: *Heparin, chemical and biological properties, clinical applications*, edited by D.A. Lane, et al, pp. 275-294. Edward Arnold, London, UK.
- Marics, I., Adelaide, J., Raybaud, F., Mattei, M.G., Coulier, F., Planche, J., de-Lapeyriere, O., and Birnbaum, D. (1989): Characterization of the HST-related FGF.6 gene, a new member of the fibroblast growth factor gene family. *Oncogene*, 4:335-340.
- Marshall, S.E., Luscombe, M., Pepper, D.S., and Holbrook, J.J. (1984): The interaction of platelet factor 4 with heparins of different chain length. *Biochim.Biophys.Acta*, 797:34-39.
- Mascarelli, F., Fuhrmann, G., and Courtois, Y. (1993): aFGF binding to low and high affinity receptors induces both aFGF and bFGF receptor dimerization. *Growth Factors*, 8:211-233.
- Masuda, Y., Yoshitake, Y., and Nishikawa, K. (1987): Secretion of DNA synthesis factor (DSF) by A431 cells that can grow in protein-free medium. *Cell Biol.Int.Rep.*, 11:359-365.
- Matsuzaki, K., Yoshitake, Y., Miyagiwa, M., Minemura, M., Tanaka, M., Sasaki, H., and Nishikawa, K. (1990): Production of basic fibroblast growth factor-like factor by cultured human cholangiocellular carcinoma cells [published erratum appears in Jpn J Cancer Res 1990 Oct;81(10):1076]. *Jpn.J.Cancer Res.*, 81:345-354.
- Matzner, Y., Bar-Ner, M., Yahalom, J., Ishai-Michaeli, R., Fuks, Z., and Vlodavsky, I. (1985): Degradation of heparan sulfate in the subendothelial extracellular matrix by a readily released heparanase from human neutrophils. Possible role in invasion through basement membranes. *J.Clin.Invest.*, 76:1306-1313.
- McCaffrey, T.A., Falcone, D.J., and Du, B. (1992): Transforming growth factor-beta 1 is a heparin-binding protein: identification of putative heparin-binding regions and isolation of heparins with varying affinity for TGF-beta 1. *J.Cell Physiol.*, 152:430-440.
- McKeehan, W.L. and Crabb, J.W. (1987): Isolation and characterization of different molecular and chromatographic forms of heparin-binding growth factor 1 from bovine brain. *Anal.Biochem.*, 164:563-569.
- McKeehan, W.L., Adams, P.S., and Rosser, M.P. (1984): Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free, primary cell culture. *Cancer Res.*, 44:1998-2010.
- McKusick, V.A. (1985): The human gene map 1 December 1984. *Clin.Genet.*, 27:207-239.



McNeil, P.L. and Ito, S. (1989): Gastrointestinal cell plasma membrane wounding and resealing in vivo. *Gastroenterology*, 96:1238-1248.

McNeil, P.L., Muthukrishnan, L., Warder, E., and D'Amore, P.A. (1989): Growth factors are released by mechanically wounded endothelial cells. *J. Cell Biol.*, 109:811-822.

Mehlman, T. and Burgess, W.H. (1990): Detection and characterization of heparin-binding proteins with a gel overlay procedure. *Anal. Biochem.*, 188:159-163.

Mergia, A., Eddy, R., Abraham, J.A., Fiddes, J.C., and Shows, T.B. (1986): The genes for basic and acidic fibroblast growth factors are on different human chromosomes. *Biochem. Biophys. Res. Commun.*, 138:644-651.

Mescher, A.L. and Gospodarowicz, D. (1979): Mitogenic effect of a growth factor derived from myelin on denervated regenerates of newt forelimbs. *J. Exp. Zool.*, 207:497-503.

Miki, T., Fleming, T.P., Bottaro, D.P., Rubin, J.S., Ron, D., and Aaronson, S.A. (1991): Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. *Science*, 251:72-75.

Milner, P.G., Li, Y.S., Hoffman, R.M., Kodner, C.M., Siegel, N.R., and Deuel, T.F. (1989): A novel 17 kD heparin-binding growth factor (HBGF-8) in bovine uterus: purification and N-terminal amino acid sequence. *Biochem. Biophys. Res. Commun.*, 165:1096-1103.

Miyagawa, K., Sakamoto, H., Yoshida, T., Yamashita, Y., Mitsui, Y., Furusawa, M., Maeda, S., Takaku, F., Sugimura, T., and Terada, M. (1988): hst-1 transforming protein: expression in silkworm cells and characterization as a novel heparin-binding growth factor. *Oncogene*, 3:383-389.

Miyamoto, M., Naruo, K.-I., Seko, C., Matsumoto, S., Kondo, T., and Kurokawa, T. (1993): Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family which has a unique secretion property. *Mol. Cell. Biol.*, 13:4251-4259.

Moenner, M., Chevallier, B., Badet, J., and Barritault, D. (1986): Evidence and characterization of the receptor to eye-derived growth factor I, the retinal form of basic fibroblast growth factor, on bovine epithelial lens cells. *Proc. Natl. Acad. Sci. U.S.A.*, 83:5024-5028.

Moenner, M., Magnaldo, I., L'Allemain, G., Barritault, D., and Pouyssegur, J. (1987): Early and late mitogenic events induced by FGF on bovine epithelial lens cells are not triggered by hydrolysis of polyphosphoinositides. *Biochem. Biophys. Res. Commun.*, 146:32-40.

Mohammadi, M., Honegger, A.M., Rotin, D., Fischer, R., Bellot, F., Li, W., Dionne, C.A., Jaye, M., Rubinstein, M., and Schlessinger, J. (1991): A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor



receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. *Mol.Cell Biol.*, 11:5068-5078.

Montesano, R., Vassalli, J.D., Baird, A., Guillemin, R., and Orci, L. (1986): Basic fibroblast growth factor induces angiogenesis in vitro. *Proc.Natl.Acad.Sci.U.S.A.*, 83:7297-7301.

Moore, R., Casey, G., Brookes, S., Dixon, M., Peters, G., and Dickson, C. (1986): Sequence, topography and protein coding potential of mouse int-2: a putative oncogene activated by mouse mammary tumour virus. *EMBO J.*, 5:919-924.

Moore, S., Pepper, D.S., and Cash, J.D. (1975): Platelet antiheparin activity. The isolation and characterisation of platelet factor 4 released from thrombin-aggregated washed human platelets and its dissociation into subunits and the isolation of membrane-bound antiheparin activity. *Biochim.Biophys.Acta*, 379:370-384.

Moscatelli, D. (1987): High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J.Cell Physiol.*, 131:123-130.

Moscatelli, D. (1988): Metabolism of receptor-bound and matrix-bound basic fibroblast growth factor by bovine capillary endothelial cells. *J.Cell Biol.*, 107:753-759.

Moscatelli, D. and Quarto, N. (1989): Transformation of NIH 3T3 cells with basic fibroblast growth factor or the hst/K-fgf oncogene causes downregulation of the fibroblast growth factor receptor: reversal of morphological transformation and restoration of receptor number by suramin. *J.Cell Biol.*, 109:2519-2527.

Moscatelli, D., Presta, M., and Rifkin, D.B. (1986): Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. *Proc.Natl.Acad.Sci.U.S.A.*, 83:2091-2095.

Mosley, B., Beckmann, M.P., March, C.J., Idzerda, R.L., Gimpel, S.D., VandenBos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., and et-al, (1989): The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms. *Cell*, 59:335-348.

Mueller, S.N., Thomas, K.A., Di-Salvo, J., and Levine, E.M. (1989): Stabilization by heparin of acidic fibroblast growth factor mitogenicity for human endothelial cells in vitro. *J.Cell Physiol.*, 140:439-448.

Murano, G., Williams, L., Miller-Anderson, M., Aronson, D.L., King, C. (1980) Some properties of antithrombin-III and its concentration in human plasma. *Thromb. Res.*, 18:259-262.

Myers, C., Trepel, J., Sartor, O., Cooper, M., Ranson, M., Toko, T., Linehan, M.W. (1993) Antigrowth factor strategies. *Cancer*, 71:1172-1178.



- Nader, H.B., Ferreira, T.M., Toma, L., Chavante, S.F., Dietrich, C.P., Casu, B., and Torri, G. (1988): Maintenance of heparan sulfate structure throughout evolution: chemical and enzymic degradation, and  $^{13}\text{C}$ -n.m.r.-spectral evidence. *Carbohydr. Res.*, 184:292-300.
- Nagao, Y. and Nishikawa, K. (1989): Basic fibroblast growth factor, albumin, and transferrin purified from rat rhodamine fibrosarcoma tissue are all essential for growth of primary tumor cells from the same tissue in serum-free medium. *In Vitro Cell Dev. Biol.*, 25:873-880.
- Nakamura, T., Teramoto, H., and Ichihara, A. (1986): Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. *Proc. Natl. Acad. Sci. U.S.A.*, 83:6489-6493.
- Nakanishi, Y., Kihara, K., Mizuno, K., Masamune, Y., Yoshitake, Y., and Nishikawa, K. (1992): Direct effect of basic fibroblast growth factor on gene transcription in a cell-free system. *Proc. Natl. Acad. Sci. U.S.A.*, 89:5216-5220.
- Naruo, K., Seko, C., Kuroshima, K., Matsutani, E., Sasada, R., Kondo, T., and Kurokawa, T. (1993): Novel secretory heparin-binding factors from human glioma cells (glia-activating factors) involved in glial cell growth. Purification and biological properties. *J. Biol. Chem.*, 268:2857-2864.
- Neufeld, G. and Gospodarowicz, D. (1985): The identification and partial characterization of the fibroblast growth factor receptor of baby hamster kidney cells. *J. Biol. Chem.*, 260:13860-13868.
- Neufeld, G. and Gospodarowicz, D. (1986): Basic and acidic fibroblast growth factors interact with the same cell surface receptors. *J. Biol. Chem.*, 261:5631-5637.
- Neufeld, G., Gospodarowicz, D., Dodge, L., and Fujii, D.K. (1987): Heparin modulation of the neurotropic effects of acidic and basic fibroblast growth factors and nerve growth factor on PC12 cells. *J. Cell Physiol.*, 131:131-140.
- Nguyen, C., Roux, D., Mattei, M.-G., deLapeyriere, O., Goldfarb, M., Birnbaum, D., and Jordan, B.R. (1988): The FGF-related oncogenes hst and int.2, and the bcl.1 locus are contained within one megabase in band q13 of chromosome 11, while the fgf.5 oncogene maps to 4q21. *Oncogene*, 3:703-708.
- Nishi, N., Matuo, Y., Muguruma, Y., Yoshitake, Y., Nishikawa, K., and Wada, F. (1985): A human prostatic growth factor (hPGF): partial purification and characterization. *Biochem. Biophys. Res. Commun.*, 132:1103-1109.
- Niswander, L. and Martin, G.R. (1992): Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development*, 114:755-768.
- Niswander, L. and Martin, G.R. (1993): FGF-4 and BMP-2 have opposite effects on limb growth. *Nature*, 361:68-71.



Nugent, M.A. and Edelman, E.R. (1992): Kinetics of basic fibroblast growth factor binding to its receptor and heparan sulfate proteoglycan: a mechanism for cooperativity. *Biochemistry*, 31:8876-8883.

Nurcombe, V., Ford, M.D., Wildschut, J.A., and Bartlett, P.F. (1993): Developmental regulation of neural response to FGF-1 and FGF-2 by heparan sulfate proteoglycan. *Science*, 260:103-106.

O'Neill, H.C. and Parish, C.R. (1983): A rapid, automated colorimetric assay for measuring antibody binding to cell surface antigens. *J.Immunol.Methods*, 64:257-268.

Ogren, S. and Lindahl, U. (1971): Degradation of heparin in mouse mastocytoma tissue. *Biochem.J.*, 125:1119-1129.

Olwin, B.B. and Hauschka, S.D. (1986): Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. *Biochemistry*, 25:3487-3492.

Olwin, B.B. and Hauschka, S.D. (1989): Cell type and tissue distribution of the fibroblast growth factor receptor. *J.Cell Biochem.*, 39:443-454.

Olwin, B.B., Burrus, L.W., Zuber, M.E., and Lueddecke, B. (1991): Characterization of a non-tyrosine kinase FGF-binding protein. *Ann.N.Y.Acad.Sci.*, 638:195-203.

Olwin, B.B. and Rapraeger, A. (1992): Repression of myogenic differentiation by aFGF, bFGF, and K-FGF is dependent on cellular heparan sulfate. *J.Cell Biol.*, 118:631-639.

Orlidge, A. and D'Amore, P.A. (1986): Cell specific effects of glycosaminoglycans on the attachment and proliferation of vascular wall components. *Microvasc.Res.*, 31:41-53.

Ornitz, D.M. and Leder, P. (1992): Ligand specificity and heparin dependence of fibroblast growth factor receptors 1 and 3. *J.Biol.Chem.*, 267:16305-16311.

Ornitz, D.M., Yayon, A., Flanagan, J.G., Svahn, C.M., Levi, E., and Leder, P. (1992): Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol.Cell Biol.*, 12:240-247.

Ortega, S., Schaeffer, M.T., Soderman, D., DiSalvo, J., Linemeyer, D.L., Gimenez-Gallego, G., and Thomas, K.A. (1991): Conversion of cysteine to serine residues alters the activity, stability, and heparin dependence of acidic fibroblast growth factor. *J.Biol.Chem.*, 266:5842-5846.

Owen, W.G. (1975): Evidence for the formation of an ester between thrombin and heparin cofactor. *Biochim.Biophys.Acta*, 405:380-387.



- Pardee, A.B., Campisi, J., and Croy, R.G. (1985): *Cold Spring Harbor Conference- Cancer Cells*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Paris, S. and Pouyssegur, J. (1991): Mitogenic effects of fibroblast growth factors in cultured fibroblasts. Interaction with the G-protein-mediated signaling pathways. *Ann.N.Y.Acad.Sci.*, 638:139-148.
- Partanen, J., Makela, T.P., Eerola, E., Korhonen, J., Hirvonen, H., Claesson-Welsh, L., and Alitalo, K. (1991): FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. *EMBO J.*, 10:1347-1354.
- Patstone, G., Pasquale, E.B., and Maher, P.A. (1993): Different members of the fibroblast growth factor receptor family are specific to distinct cell types in the developing chicken embryo. *Dev.Biol.*, 155:107-123.
- Pepper, M.S. and Meda, P. (1992): Basic fibroblast growth factor increases junctional communication and connexin 43 expression in microvascular endothelial cells. *J.Cell Physiol.*, 153:196-205.
- Peters, G., Brookes, S., Smith, R., and Dickson, C. (1983): Tumorigenesis by mouse mammary tumor virus: evidence for a common region for provirus integration in mammary tumors. *Cell*, 33:369-377.
- Peters, K.G., Werner, S., Chen, G., and Williams, L.T. (1992a): Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development*, 114:233-243.
- Peters, K.G., Marie, J., Wilson, E., Ives, H.E., Escobedo, J., Del-Rosario, M., Mirda, D., and Williams, L.T. (1992b): Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and  $Ca^{2+}$  flux but not mitogenesis. *Nature*, 358:678-681.
- Petitou, M., Lormeau, J.C., and Choay, J. (1988): Interaction of heparin and antithrombin III. The role of O-sulfate groups. *Eur.J.Biochem.*, 176:637-640.
- Piepkorn, M., Hovingh, P., and Linker, A. (1989): Glycosaminoglycan free chains. External plasma membrane components distinct from the membrane proteoglycans. *J.Biol.Chem.*, 264:8662-8669.
- Poole, A.R. (1986): Proteoglycans in health and disease: structures and functions. *Biochem.J.*, 236:1-14.
- Powell, J.A. (1969): Analysis of histogenesis and regenerative ability of denervated forelimb regenerates of *Triturus viridescens*. *J.Exp.Zool.*, 170:125-147.
- Prats, H., Kaghad, M., Prats, A.C., Klagsbrun, M., Lelias, J.M., Liauzun, P., Chalon, P., Tauber, J.P., Amalric, F., Smith, J.A., and et-al, (1989): High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. *Proc.Natl.Acad.Sci.U.S.A*, 86:1836-1840.



- Presta, M., Moscatelli, D., Joseph-Silverstein, J., and Rifkin, D.B. (1986): Purification from a human hepatoma cell line of a basic fibroblast growth factor-like molecule that stimulates capillary endothelial cell plasminogen activator production, DNA synthesis, and migration. *Mol. Cell Biol.*, 6:4060-4066.
- Presta, M., Rusnati, M., Urbinati, C., Sommer, A., and Ragnotti, G. (1991): Biologically active synthetic fragments of human basic fibroblast growth factor (bFGF): identification of two Asp-Gly-Arg-containing domains involved in the mitogenic activity of bFGF in endothelial cells. *J. Cell Physiol.*, 149:512-524.
- Prestrelski, S.J., Fox, G.M., and Arakawa, T. (1992): Binding of heparin to basic fibroblast growth factor induces a conformational change. *Arch. Biochem. Biophys.*, 293:314-319.
- Quarto, N., Finger, F.P., and Rifkin, D.B. (1991): The NH2-terminal extension of high molecular weight bFGF is a nuclear targeting signal. *J. Cell Physiol.*, 147:311-318.
- Ramsden, L. and Rider, C.C. (1992): Selective and differential binding of interleukin (IL)-1alpha, IL-1beta, IL-2 and IL-6 to glycosaminoglycans. *J. Immunol.*, 22:3027-3031.
- Rapp, U.R. (1991): Role of Raf-1 serine/threonine protein kinase in growth factor signal transduction. *Oncogene*, 6:495-500.
- Rapraeger, A., Jalkanen, M., and Bernfield, M. (1987): Integral membrane proteoglycans as matrix receptors: role in cytoskeleton and matrix assembly at the epithelial cell surface. In: *Biology of Extracellular Matrix: A series. Biology of Proteoglycans*, edited by T.N. Wright, et al, pp. 129-154. Orlando Academic,
- Rapraeger, A.C., Krufka, A., and Olwin, B.B. (1991): Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. *Science*, 252:1705-1708.
- Redini, F., Moczar, E., Antoine, E., and Poupon, M.F. (1989): Binding and internalization of exogenous glycosaminoglycans in weakly and highly metastatic rhabdomyosarcoma cells. *Biochim. Biophys. Acta*, 991:359-366.
- Reid, H.H., Wilks, A.F., and Bernard, O. (1990): Two forms of the basic fibroblast growth factor receptor-like mRNA are expressed in the developing mouse brain. *Proc. Natl. Acad. Sci. U.S.A.*, 87:1596-1600.
- Renko, M., Quarto, N., Morimoto, T., and Rifkin, D.B. (1990): Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. *J. Cell Physiol.*, 144:108-114.
- Rifkin, D.B. and Moscatelli, D. (1989): Recent developments in the cell biology of basic fibroblast growth factor. *J. Cell Biol.*, 109:1-6.



Rifkin, D.B., Quarto, N., Mignatti, P., Bizik, J., and Moscatelli, D. (1991): New observations on the intracellular localization and release of bFGF. *Ann.N.Y.Acad.Sci.*, 638:204-206.

Roberts, R., Gallagher, J., Spooncer, E., Allen, T.D., Bloomfield, F., and Dexter, T.M. (1988): Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. *Nature*, 332:376-378.

Rodan, S.B., Wesolowski, G., Thomas, K., and Rodan, G.A. (1987): Growth stimulation of rat calvaria osteoblastic cells by acidic fibroblast growth factor. *Endocrinology*, 121:1917-1923.

Rogelj, S., Weinberg, R.A., Fanning, P., and Klagsbrun, M. (1988): Basic fibroblast growth factor fused to a signal peptide transforms cells. *Nature*, 331:173-175.

Rosenbaum, J., Tobelem, G., Molho, P., Barzu, T., and Caen, J.P. (1986): Modulation of endothelial cells growth induced by heparin. *Cell Biol.Int.Rep.*, 10:437-446.

Rosenberg, R.D. and Damus, P.S. (1973): The purification and mechanism of action of human antithrombin-heparin cofactor. *J.Biol.Chem.*, 248:6490-6505.

Rosengart, T.K., Johnson, W.V., Friesel, R., Clark, R., and Maciag, T. (1988): Heparin protects heparin-binding growth factor-I from proteolytic inactivation in vitro. *Biochem.Biophys.Res.Comm.*, 152:432-440.

Rubin, J.S., Osada, H., Finch, P.W., Taylor, W.G., Rudikoff, S., and Aaronson, S.A. (1989): Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc.Natl.Acad.Sci.U.S.A.*, 86:802-806.

Ruoslahti, E. and Pierschbacher, M.D. (1986): Arg-Gly-Asp: a versatile cell recognition signal. *Cell*, 44:517-518.

Ruoslahti, E. and Pierschbacher, M.D. (1987): New perspectives in cell adhesion: RGD and integrins. *Science*, 238:491-497.

Rusnati, M., Urbinati, C., and Presta, M. (1993): Internalization of basic fibroblast growth factor (bFGF) in cultured endothelial cells: role of the low affinity heparin-like bFGF receptors. *J.Cell Physiol.*, 154:152-161.

Ruta, M., Howk, R., Ricca, G., Drohan, W., Zabelshansky, M., Laureys, G., Barton, D.E., Franke, U., Schlessinger, J., and Givol, D. (1988): A novel protein tyrosine kinase gene whose expression is modulated during endothelial cell differentiation. *Oncogene*, 3:9-15.

Rydel, R.E. and Greene, L.A. (1987): Acidic and basic fibroblast growth factors promote stable neurite outgrowth and neuronal differentiation in cultures of PC12 cells. *J.Neurosci.*, 7:3639-3653.



Rylatt, D.B., Sia, D.Y., Mundy, J.P., and Parish, C.R. (1981): Autorosette inhibition factor: isolation and properties of the human plasma protein. *Eur.J.Biochem.*, 119:641-646.

Sakamoto, H., Mori, M., Taira, M., Yoshida, T., Matsukawa, S., Shimizu, K., Sekiguchi, M., Terada, M., and Sugimura, T. (1986): Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosa. *Proc.Natl.Acad.Sci.U.S.A.*, 83:3997-4001.

Sakamoto, H., Yoshida, T., Nakakuki, M., Odagiri, H., Miyagawa, K., Sugimura, T., and Terada, M. (1988): Cloned hst gene from normal human leukocyte DNA transforms NIH3T3 cells. *Biochem.Biophys.Res.Comm.*, 151:965-972.

Saksela, O., Moscatelli, D., Sommer, A., and Rifkin, D.B. (1988): Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. *J.Cell Biol.*, 107:743-751.

Saksela, O. and Rifkin, D.B. (1990): Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. *J.Cell Biol.*, 110:767-775.

4.

Satoh, H., Shino, A., Inatomi, N., Nagaya, H., Sato, F., and Szabo, S. (1991): Effects of rhbFGF mutein CS23 (TGP-580) on the healing of gastric ulcers induced by acetic acid in rats. *Gastroenterology*, 100:A155:

Schreiber, A.B., Kenney, J., Kowalski, W.J., Friesel, R., Mehlman, T., and Maciag, T. (1985): Interaction of endothelial cell growth factor with heparin: characterization by receptor and antibody recognition. *Proc.Natl.Acad.Sci.U.S.A.*, 82:6138-6142.

Schubert, D., Ling, N., and Baird, A. (1987): Multiple influences of a heparin-binding growth factor on neuronal development. *J.Cell Biol.*, 104:635-643.

Schweigerer, L., Neufeld, G., Friedman, J., Abraham, J.A., Fiddes, J.C., and Gospodarowicz, D. (1987a): Basic fibroblast growth factor: production and growth stimulation in cultured adrenal cortex cells. *Endocrinology*, 120:796-800.

Schweigerer, L., Neufeld, G., Mergia, A., Abraham, J.A., Fiddes, J.C., and Gospodarowicz, D. (1987b): Basic fibroblast growth factor in human rhabdomyosarcoma cells: implications for the proliferation and neovascularization of myoblast-derived tumors. *Proc.Natl.Acad.Sci.U.S.A.*, 84:842-846.

Schweigerer, L., Neufeld, G., Friedman, J., Abraham, J.A., Fiddes, J.C., and Gospodarowicz, D. (1987c): Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. *Nature*, 325:257-259.

Seed, J., Olwin, B.B., and Hauschka, S.D. (1988): Fibroblast growth factor levels in the whole embryo and limb bud during chick development. *Dev.Biol.*, 128:50-57.



Segal, S.S. and Duling, B.R. (1986): Flow control among microvessels coordinated by intercellular conduction. *Science*, 234:868-870.

Segal, S.S. and Duling, B.R. (1987): Propagation of vasodilation in resistance vessels of the hamster: development and review of a working hypothesis. *Circ.Res.*, 61:II20-II25.

Segal, S.S. and Duling, B.R. (1989): Conduction of vasomotor responses in arterioles: a role for cell-to-cell coupling? *Am.J.Physiol.*, 256:H838-H845.

Seifert, R.A., Hart, C.E., Phillips, P.E., Forstrom, J.W., Ross, R., Murray, M.J., and Bowen-Pope, D.F. (1989): Two different subunits associate to create isoform-specific platelet-derived growth factor receptors. *J.Biol.Chem.*, 264:8771-8778.

Senior, R.M., Griffin, G.L., Huang, J.S., Walz, D.A., and Deuel, T.F. (1983): Chemotactic activity of platelet alpha granule proteins for fibroblasts. *J.Cell Biol.*, 96:382-385.

Senior, R.M., Huang, S.S., Griffin, G.L., and Huang, J.S. (1986): Brain-derived growth factor is a chemoattractant for fibroblasts and astroglial cells. *Biochem.Biophys.Res.Comm.*, 141:67-72.

Seno, M., Sasada, R., Kurokawa, T., and Igarashi, K. (1990): Carboxyl-terminal structure of basic fibroblast growth factor significantly contributes to its affinity for heparin. *Eur.J.Biochem.*, 188:239-245.

Serrero, G. and Khoo, J.C. (1982): An in vitro model to study adipose differentiation in serum-free medium. *Anal.Biochem.*, 120:351-359.

Sharpe, R.J., Byers, H.R., Scott, C.F., Bauer, S.I., and Maione, T.E. (1990): Growth inhibition of murine melanoma and human colon carcinoma by recombinant human platelet factor 4. *J.Natl.Cancer Inst.*, 82:848-853.

Shatsky, M., Saigo, K., Burdach, S., Leung, L.L., and Levitt, L.J. (1989): Histidine-rich glycoprotein blocks T cell rosette formation and modulates both T cell activation and immunoregulation. *J.Biol.Chem.*, 264:8254-8259.

Shing, Y. (1988): Heparin-copper bioaffinity chromatography of fibroblast growth factors. *J.Biol.Chem.*, 263:9059-9062.

Shing, Y., Folkman, J., Murray, J., and Klagsbrun, M. (1983): Purification by affinity chromatography on heparin-Sepharose of a growth factor that stimulates capillary endothelial cell proliferation. *J.Cell Biol.*, 97:395a.(Abstract)

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J., and Klagsbrun, M. (1984): Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science*, 223:1296-1299.

Shishido, E., Higashijima, S.-I., Emori, Y., and Saigo, K. (1993): Two FGF-receptor homologues of Drosophila: one is expressed in mesodermal primordium in early embryos. *Development*, 117:751-761.



Shiurba, R.A., Jing, N., Sakakura, T., and Godsave, S.F. (1991): Nuclear translocation of fibroblast growth factor during *Xenopus* mesoderm induction. *Development*, 113:487-493.

Sievers, J., Hausmann, B., Unsicker, K., and Berry, M. (1987): Fibroblast growth factors promote the survival of adult rat retinal ganglion cells after transection of the optic nerve. *Neurosci.Lett.*, 76:157-162.

Silbert, J.E. (1982): Structure and metabolism of proteoglycans and glycosaminoglycans. *J.Invest.Dermatol.*, 79 Suppl 1:31s-37s.

Silverstein, R.L., Nachman, R.L., Leung, L.L., and Harpel, P.C. (1985): Activation of immobilized plasminogen by tissue activator. Multimolecular complex formation. *J.Biol.Chem.*, 260:10346-10352.

Singer, M. (1974): Trophic functions of the neuron. VI. Other trophic systems. Neurotrophic control of limb regeneration in the newt. *Ann.N.Y.Acad.Sci.*, 228:308-322.

Skerrett, P.J. (1991): "Matrix algebra" heals life's wounds [news]. *Science*, 252:1064-1066.

Slack, J.M., Darlington, B.G., Heath, J.K., and Godsave, S.F. (1987): Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. *Nature*, 326:197-200.

Sommer, A. and Rifkin, D.B. (1989): Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. *J.Cell Physiol.*, 138:215-220.

Stiles, C.D., Capone, G.T., Scher, C.D., Antoniades, H.N., Van-Wyk, J.J., and Pledger, W.J. (1979): Dual control of cell growth by somatomedins and platelet-derived growth factor. *Proc.Natl.Acad.Sci.U.S.A.*, 76:1279-1283.

Sullivan, R. and Klagsbrun, M. (1985): Purification of cartilage-derived growth factor by heparin affinity chromatography. *J.Biol.Chem.*, 260:2399-2403.

Suzuki, H.R., Sakamoto, H., Yoshida, T., Sugimura, T., Terada, M., and Solursh, M. (1992): Localization of HstI transcripts to the apical ectodermal ridge in the mouse embryo. *Dev.Biol.*, 150:219-222.

Suzuki, K., Kamel, T., Hakamata, Y., Kikukawa, K., Shiota, K., and Takahashi, M. (1991): Basic fibroblast growth factor-like substance in nuclei of male germ cells undergoing meiosis. *Proc.Soc.Exp.Biol.Med.*, 198:728-731.

Taira, M., Yoshida, T., Miyagawa, K., Sakamoto, H., Terada, M., and Sugimura, T. (1987): cDNA sequence of human transforming gene hst and identification of the coding sequence required for transforming activity. *Proc.Natl.Acad.Sci.U.S.A.*, 84:2980-2984.



- Takahashi, J.A., Mori, H., Fukumoto, M., Igarashi, K., Jaye, M., Oda, Y., Kikuchi, H., and Hatanaka, M. (1990): Gene expression of fibroblast growth factors in human gliomas and meningiomas: demonstration of cellular source of basic fibroblast growth factor mRNA and peptide in tumor tissues. *Proc.Natl.Acad.Sci.U.S.A.*, 87:5710-5714.
- Tanaka, A., Miyamoto, K., Minamino, N., Takeda, M., Sato, B., Matsuo, H., and Matsumoto, K. (1992): Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. *Proc.Natl.Acad.Sci.U.S.A.*, 89:8928-8932.
- Tarsio, J.F., Rubin, N.A., Russell, P., Gregerson, D.S., and Reid, T.W. (1983): Growth-stimulatory effects of retinoblastoma-derived growth factors and other mitogens on Nakano mouse lens epithelial cells. *Exp.Cell Res.*, 146:71-78.
- Taylor, S. and Folkman, J. (1982): Protamine is an inhibitor of angiogenesis. *Nature*, 297:307-312.
- Taylor, R.L., Shively, J.E., and Conrad, H.E. (1976): Stoichiometric reduction of uronic acid carboxyl groups in polysaccharides. In: *Methods in Carbohydrate Chemistry*, pp. 149-151. *Methods in Carbohydrate Chemistry*,
- Terranova, V.P., DiFlorio, R., Lyall, R.M., Hic, S., Friesel, R., and Maciag, T. (1985): Human endothelial cells are chemotactic to endothelial cell growth factor and heparin. *J.Cell Biol.*, 101:2330-2334.
- Tessler, S. and Neufeld, G. (1990): Basic fibroblast growth factor accumulates in the nuclei of various bFGF-producing cell types. *J.Cell.Physiol.*, 145:310-317.
- Thomas, K.A. and Gimenez-Gallego, G. (1986): Fibroblast growth factors: broad spectrum mitogens with potent angiogenic activity. *TIBS*, 11:81-84.
- Thomas, K.A., Riley, M.C., Lemmon, S.K., Baglan, N.C., and Bradshaw, R.A. (1980): Brain fibroblast growth factor: nonidentity with myelin basic protein fragments. *J.Biol.Chem.*, 255:5517-5520.
- Thomas, K.A., Rios-Candelore, M., and Fitzpatrick, S. (1984): Purification and characterization of acidic fibroblast growth factor from bovine brain. *Proc.Natl.Acad.Sci.U.S.A.*, 81:357-361.
- Thomas, K.A., Rios-Candelore, M., Gimenez-Gallego, G., DiSalvo, J., Bennett, C., Rodkey, J., and Fitzpatrick, S. (1985): Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial cell mitogen with sequence homology to interleukin 1. *Proc.Natl.Acad.Sci.U.S.A.*, 82:6409-6413.
- Thornton, S.C., Mueller, S.N., and Levine, E.M. (1983): Human endothelial cells: use of heparin in cloning and long-term serial cultivation. *Science*, 222:623-625.



Togari, A., Baker, D., Dickens, G., and Guroff, G. (1983): The neurite-promoting effect of fibroblast growth factor on PC12 cells. *Biochem. Biophys. Res. Commun.*, 114:1189-1193.

Togari, A., Dickens, G., Kuzuya, H., and Guroff, G. (1985): The effect of fibroblast growth factor on PC12 cells. *J. Neurosci.*, 5:307-316.

Tomomura, M., Kadomatsu, K., Nakamoto, M., Muramatsu, H., Kondoh, H., Imagawa, K., and Muramatsu, T. (1990): A retinoic acid responsive gene, MK, produces a secreted protein with heparin binding activity. *Biochem. Biophys. Res. Commun.*, 171:603-609.

Trowell, O.A., Chir, B., and Willmer, E.N. (1939): Studies on the growth of tissues in vitro. VI. The effects of some tissue extracts on the growth of periosteal fibroblasts. *J. Exp. Biol.*, 16:60-70.

Tsuda, T., Kaibuchi, K., West, B., and Takai, Y. (1985): Involvement of  $Ca^{2+}$  in platelet-derived growth factor-induced expression of c-myc oncogene in Swiss 3T3 fibroblasts. *FEBS Lett.*, 187:43-46.

Tsuda, H., Hirohashi, S., Shimosato, Y., Hirota, T., Tsugane, S., Yamamoto, H., Miyajima, N., Toyoshima, K., Yamamoto, T., Yokota, J., and et-al, (1989): Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: hst-1/int-2 and c-erbB-2/ear-1. *Cancer Res.*, 49:3104-3108.

Turnbull, J.E., Fernig, D.G., Ke, Y., Wilkinson, M.C., and Gallagher, J.T. (1992): Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate. *J. Biol. Chem.*, 267:10337-10341.

Turnbull, J.E. and Gallagher, J.T. (1991): Distribution of iduronate 2-sulphate residues in heparan sulphate. Evidence for an ordered polymeric structure. *Biochem. J.*, 273:553-559.

Tyrrell, D.J., Ishihara, M., Rao, N., Horne, A., Kiefer, M.C., Stauber, G.B., Lam, L.H., and Stack, R.J. (1993): Structure and biological activities of a heparin-derived hexasaccharide with high affinity for basic fibroblast growth factor. *J. Biol. Chem.*, 268:4684-4689.

Ueno, N., Baird, A., Esch, F., Shimasaki, S., Ling, N., and Guillemín, R. (1986): Purification and partial characterization of a mitogenic factor from bovine liver: structural homology with basic fibroblast growth factor. *Regul. Pept.*, 16:135-145.

Ueno, N., Baird, A., Esch, F., Ling, N., and Guillemín, R. (1987): Isolation and partial characterization of basic fibroblast growth factor from bovine testis. *Mol. Cell Endocrinol.*, 49:189-194.

Ueno, H., Gunn, M., Dell, K., Tseng, A.J., and Williams, L. (1992): A truncated form of fibroblast growth factor receptor 1 inhibits signal transduction by multiple types of fibroblast growth factor receptor. *J. Biol. Chem.*, 267:1470-1476.



Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.C., Tsubokawa, M., and et-al, (1985): Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature*, 313:756-761.

Ullrich, A., Gray, A., Tam, A.W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le-Bon, T., Kathuria, S., Chen, E., and et-al, (1986): Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J.*, 5:2503-2512.

Ullrich, A. and Schlessinger, J. (1990): Signal transduction by receptors with tyrosine kinase activity. *Cell*, 61:203-212.

Unsicker, K., Reichert-Preibsch, H., Schmidt, R., Pettmann, B., Labourdette, G., and Sensenbrenner, M. (1987): Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central nervous system neurons. *Proc.Natl.Acad.Sci.U.S.A*, 84:5459-5463.

Vannuchi, S., Pasquali, F., Chiarugi, V., and Ruggiero, M. (1986): Internalization and metabolism of endogenous heparin. *Biochem.Biophys.Res.Comm.*, 140:294-301.

Vannucchi, S., Pasquali, F., Porciatti, F., Chiarugi, V., Magnelli, L., and Bianchini, P. (1988): Binding, internalization and degradation of heparin and heparin fragments by cultured endothelial cells. *Thromb.Res.*, 49:373-383.

Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J., and Klagsbrun, M. (1987a): Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *Proc.Natl.Acad.Sci.U.S.A*, 84:2292-2296.

Vlodavsky, I., Fridman, R., Sullivan, R., Sasse, J., and Klagsbrun, M. (1987b): Aortic endothelial cells synthesize basic fibroblast growth factor which remains cell associated and platelet-derived growth factor-like protein which is secreted. *J.Cell Physiol.*, 131:402-408.

Vlodavsky, I., Bar-Shavit, R., Ishai-Michaeli, R., Bashkin, P., and Fuks, Z. (1991a): Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? *Trends Biochem.Sci.*, 16:268-271.

Vlodavsky, I., Bashkin, P., Ishai-Michaeli, R., Chajek-Shaul, T., Bar-Shavit, R., Haimovitz-Friedman, A., Klagsbrun, M., and Fuks, Z. (1991b): Sequestration and release of basic fibroblast growth factor. *Ann.N.Y.Acad.Sci.*, 638:207-220.

Vlodavsky, I., Fuks, Z., Ishai-Michaeli, R., Bashkin, P., Levi, E., Korner, G., Bar-Shavit, R., and Klagsbrun, M. (1991c): Extracellular matrix-resident basic fibroblast growth factor: implication for the control of angiogenesis. *J.Cell.Biochem.*, 45:167-176.



- Volkin, D.B., Tsai, P.K., Dabora, J.M., Gress, J.O., Burke, C.J., Linhardt, R.J., and Middaugh, C.R. (1993): Physical stabilization of acidic fibroblast growth factor by polyanions. *Arch.Biochem.Biophys.*, 300:30-41.
- Wada, A., Sakamoto, H., Katoh, O., Yoshida, T., Yokota, J., Little, P.F., Sugimura, T., and Terada, M. (1988): Two homologous oncogenes, HST1 and INT2, are closely located in human genome. *Biochem.Biophys.Res.Commun.*, 157:828-835.
- Walicke, P. (1989): Novel neurotrophic factors, receptors and oncogenes. *Ann.Rev.Neurosci.*, 12:103-126.
- Walicke, P., Cowan, W.M., Ueno, N., Baird, A., and Guillemin, R. (1986): Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc.Natl.Acad.Sci.U.S.A.*, 83:3012-3016.
- Walicke, P.A. and Baird, A. (1988): Neurotrophic effects of basic and acidic fibroblast growth factors are not mediated through glial cells. *Brain Res.*, 468:71-79.
- Walicke, P.A. and Baird, A. (1991): Internalization and processing of basic fibroblast growth factor by neurons and astrocytes. *J.Neurosci.*, 11:2249-2258.
- Walicke, P.A., Feige, J.J., and Baird, A. (1989): Characterization of the neuronal receptor for basic fibroblast growth factor and comparison to receptors on mesenchymal cells. *J.Biol.Chem.*, 264:4120-4126.
- Weiner, H.L. and Swain, J.L. (1989): Acidic fibroblast growth factor mRNA is expressed by cardiac myocytes in culture and the protein is localized to the extracellular matrix. *Proc.Natl.Acad.Sci.U.S.A.*, 86:2683-2687.
- Weisgraber, K.H., Rall, S.C.J., Mahley, R.W., Milne, R.W., Marcel, Y.L., and Sparrow, J.T. (1986): Human apolipoprotein E. Determination of the heparin binding sites of apolipoprotein E3. *J.Biol.Chem.*, 261:2068-2076.
- Werner, S., Duan, D.S., de-Vries, C., Peters, K.G., Johnson, D.E., and Williams, L.T. (1992): Differential splicing in the extracellular region of fibroblast growth factor receptor 1 generates receptor variants with different ligand-binding specificities. *Mol.Cell Biol.*, 12:82-88.
- Westall, F.C., Lennon, V.A., and Gospodarowicz, D. (1978): Brain-derived fibroblast growth factor: identity with a fragment of the basic protein of myelin. *Proc.Natl.Acad.Sci.U.S.A.*, 75:4675-4678.
- Whalen, G.F., Shing, Y., and Folkman, J. (1989): The fate of intravenously administered bFGF and the effect of heparin. *Growth Factors*, 1:157-164.
- Wice, B., Milbrandt, J., and Glaser, L. (1987): Control of muscle differentiation in BC3H1 cells by fibroblast growth factor and vanadate. *J.Biol.Chem.*, 262:1810-1817.



- Wilkinson, D.G., Bhatt, S., and McMahon, A.P. (1989): Expression pattern of the FGF-related proto-oncogene int-2 suggests multiple roles in fetal development. *Development*, 105:131-136.
- Wilks, A.F. (1993): Protein tyrosine kinase growth factor receptors and their ligands in development, differentiation, and cancer. *Adv.Cancer Res.*, 60:43-73.
- Williams, A.F. and Barclay, A.N. (1988): The immunoglobulin superfamily--domains for cell surface recognition. *Annu.Rev.Immunol.*, 6:381-405.
- Williams, L.T. (1989): Signal transduction by the platelet-derived growth factor receptor. *Science*, 243:1564-1570.
- Williams, A.F. (1991): Out of Equilibrium. *Nature*, 352:473-474
- Witte, L., Fuks, Z., Haimovitz-Friedman, A., Vlodavsky, I., Goodman, D.S., and Eldor, A. (1989): Effects of irradiation on the release of growth factors from cultured bovine, porcine, and human endothelial cells. *Cancer Res.*, 49:5066-5072.
- Wu, D.K., Maciag, T., and de-Vellis, J. (1988): Regulation of neuroblast proliferation by hormones and growth factors in chemically defined medium. *J.Cell Physiol.*, 136:367-372.
- Yarden, Y. and Ullrich, A. (1988): Growth factor receptor tyrosine kinases. *Annu.Rev.Biochem.*, 57:443-478.
- Yayon, A. and Klagsbrun, M. (1990): Autocrine transformation by chimeric signal peptide-basic fibroblast growth factor: reversal by suramin. *Proc.Natl.Acad.Sci.U.S.A*, 87:5346-5350.
- Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P., and Ornitz, D.M. (1991): Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell*, 64:841-848.
- Yoshida, M.C., Wada, M., Satoh, H., Yoshida, T., Sakamoto, H., Miyagawa, K., Yokota, J., Koda, T., Kakinuma, M., Sugimura, T., and et-al, (1988): Human HST1 (HSTF1) gene maps to chromosome band 11q13 and coamplifies with the INT2 gene in human cancer. *Proc.Natl.Acad.Sci.U.S.A*, 85:4861-4864.
- Yuen, S.N., Folkman, J., Weisz, P.B., Joullie, M.M., and Ewing, W.R. (1990): Affinity of fibroblast growth factors for beta-cyclodextrin tetradecasulfate. *Anal.Biochem.*, 185:108-111.
- Yurt, R.W., Leid, R.W.J., and Austen, K.F. (1977): Native heparin from rat peritoneal mast cells. *J.Biol.Chem.*, 252:518-521.
- Zhan, X., Bates, B., Hu, X.G., and Goldfarb, M. (1988): The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol.Cell Biol.*, 8:3487-3495.

Zhan, X., Hu, X., Friesel, R., and Maciag, T. (1993): Long term growth factor exposure and differential tyrosine phosphorylation are required for DNA synthesis in Balb/c 3T3 cells. *J.Biol.Chem.*, 268:9611-9620.

Zhang, J.D., Cousens, L.S., Barr, P.J., and Sprang, S.R. (1991): Three-dimensional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1 beta [published erratum appears in Proc Natl Acad Sci U S A 1991 Jun 15;88(12): 5477]. *Proc.Natl.Acad.Sci.U.S.A*, 88:3446-3450.

Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G.M., Arakawa, T., Hsu, B.T., and Rees, D.C. (1990): Three-dimensional structures of acidic and basic fibroblast growth factors. *Science*, 251:90-93.

Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G.M., Arakawa, T., Hsu, B.T., and Rees, D.C. (1991): Three-dimensional structures of acidic and basic fibroblast growth factors. *Science*, 251:90-93.

•